Generation of calcineurin inhibitor resistant EBV-CTLs for the treatment of post transplant lymphoma

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Declaration

I, Jennifer Brewin, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Epstein Barr Virus driven post transplant lymphoproliferative disease (PTLD) is a serious complication following either stem cell or solid organ transplantation (SOT), occurring as a result of suppressed cellular immunity. Adoptive transfer of EBV specific cytotoxic T cells (EBV-CTLs) is effective as both prophylaxis and treatment for PTLD following stem cell transplantation, but is less effective when applied to PTLD after SOT. It is likely that the continued pharmacologic immunosuppression designed to prevent graft rejection compromises the function of infused EBV-CTLs. To address this issue, we have generated calcineurin (CN) mutants that do not bind the immunosuppressants Tacrolimus (FK506) and Cyclosporin A (CsA), therefore conferring resistance to these CN inhibitors. A panel of 54 such mutants was designed, generated and screened in a Luciferase expression assay, with identification of those capable of resisting suppression with FK506, CsA or both. Subsequently, in assays utilising both the Jurkat T cell line and primary human EBV-CTL lines, mutant CNa12 conferred resistance to CsA, mutant CNa22 conferred resistance to FK506 and mutant CNb30 rendered cells resistant to both calcineurin inhibitors. EBV-CTL lines transduced with a retroviral vector encoding these mutants retained the ability to both proliferate and secrete normal levels of interferon-γ in the presence of therapeutic and supratherapeutic levels of FK506 (CNa12), CsA (CNa22), or both (CNb30). The cytotoxicity, phenotype and antigen dependence of EBV-CTL lines were unaffected by expression of mutant CN. A xenogenic murine model of PTLD was established in the $RAG2^{-/-}/\gamma c^{-/-}/C5^{-/-}$ triple knockout SCID mouse strain and the ability of EBV-CTL lines to induce tumour regression was examined. Administration of EBV-CTLs caused regression of subcutaneous LCL tumours *in vivo* in some donors. This model will be utilised in further investigations of transduced EBV-CTLs *in vivo*. The generation of EBV-CTLs that are resistant to CN inhibitors should allow effective immunotherapy in the SOT setting without risking rejection of the graft by reducing immunosuppression. Additionally this represents a generic approach to improving immunotherapy in the face of immunosuppression in other settings.

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Abbreviations

^{3}H	Hydrogen^{3} labelled Thymidine
^{51}Cr	Chromium^{51} labelled Sodium Chromate
AA	Amino acid
ADCC	Antibody Dependent Cellular Cytotoxicity
ATG	Anti-thymocyte globulin
ATG	Anti-thymocyte globulin
BART	Bam A Rightward Transcript
BFP	Blue fluorescent protein
BL	Burkitt Lymphoma
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation marker
CDC	Complement Mediated Cytotoxicity
CMV	Cytomegalovirus
CN	Calcineurin
CNS	Central Nervous System
CsA	Cyclosporin A
CTL	Cytotoxic T Lymphocyte
CyPA	Cyclophilin A
DAG	Diacyl glycerol
DLI	Donor Lymphocyte Infusion
DNA	Deoxyribonucleic Acid
EA	Early Antigen
EBER	EBV Encoded RNA
EBNA	EBV Nuclear Antigen
EBV	Epstein Barr Virus
ELISA	Enzyme Linked Immunosorbant Assay
ELISPOT	Enzyme Linked Immunosorbant Spot Assay
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FK506	Tacrolimus
FKBP12	FK506 binding protein 12
FLuc	Firefly luciferase
GALV	Gibbon ape leukaemia virus
GC	Germinal Centre
GFP	Green Fluorescent Protein
GVHD	Graft versus host disease
GVL	Graft versus Leukaemia
HIV	Human Immunodeficiency Virus
HL	Hodgkin Lymphoma

HLA	Human Leukocyte Antigen
HSC	Haematopoietic Stem Cell
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	Interferon
IL	Interleukin
IM	Infectious Mononucleosis
IRES	Internal ribosomal entry site
IVIS	*In vivo* imaging system
JAK	Janus Kinase
LCL	Lymphoblastoid Cell Line
LMP	Latent Membrane Protein
LPD	Lymphoproliferative disorder
LTR	Long Terminal Repeat
MAP	Mitogen Activated Protein
MFI	Mean fluorescence index
MHC	Major Histocompatibility Complex
MLV	Murine Leukaemia Virus
MMF	Mycophenolate Mofetil
neo^R	Neomycin
NFAT	Nuclear Factor of Activated T cells
NFKB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer Cells
NOD	Non Obese Diabetic
NPC	Nasopharyngeal Carcinoma
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIP_2	Phosphatidylinositol-5,4-bisphosphate
PKCθ	Protein kinase C θ
PLCγ1	Phospholipase C γ 1
PMA	Phorbol 12-myristate 13-acetate
PTLD	Post Transplant Lymphoproliferative Disease
RLU	Relative light units
RLuc	Renilla Luciferase
RN	Retronectin
RNA	Ribonucleic Acid
ROI	Region of interest
RV	Retrovirus
s.c.	Subcutaneous
SAP	SLAM Associated Protein
SCT	Stem Cell Transplant

SIN	Self Inactivating
SOT	Solid Organ Transplant
STAT	Signal Transducer and Activator of Transcription
TCD	T cell depletion
TCR	T cell receptor
TNF	Tumour Necrosis Factor
UT	Untransduced
VCA	Viral Capsid Antigen
WHO	World Health Authority
XLP	X-linked Lymphoproliferative Disease
X-SCID	X-linked Severe Combined Immunodeficiency

Chapter one

Introduction

1.1 Epstein Barr Virus

Following the description of Burkitt Lymphoma in 1958, Epstein, Barr and Achong identified the Epstein Barr Virus (EBV) from tumour samples (Epstein*, et al* 1964). This virus has since been identified as a gamma herpesvirus infecting over 90% of adults worldwide and classified as Human Herpesvirus 4, a member of the lymphocryptovirus genus (Kieff and Rickinson 2007). After primary exposure, EBV establishes a latent infection of resting memory B cells, with occasional sporadic production of lytic virus from the oropharyngeal cavity but no clinical symptoms in most individuals. However, despite the usual subclinical course, EBV contributes a significant burden to human health worldwide, being responsible for the development of infectious mononucleosis, found in association with several human tumours, and possibly contributing to the development of autoimmunity.

1.1.1 Characteristics of EBV

Infectious EBV particles consist of a toroid DNA core within a nuclear capsid, enclosed within the viral envelope which contains many protruding glycoproteins. These glycoproteins are involved in initiation of infection, with the most abundant, gp350/220, binding to the CD21 molecule on B lymphocytes. Binding of several gp350/220 molecules results in both aggregation and probable activation of the CD21 complex, and uptake of the viral particles via the endocytosis pathway. Glycoproteins gp42 in association with gH and gL also contribute to B cell adhesion through MHC class II binding, an interaction that is thought to be important in fusion to the endosomal membrane, resulting in entry of the viral capsid to the cytoplasm. Although B cell tropic *in vitro*, EBV is also capable of infecting epithelial cells *in vivo*, however the receptor for entry in CD21 negative epithelial cells is thus far uncharacterised (Cohen 2000, Kieff and Rickinson 2007).

EBV has a linear DNA genome of approximately 180kb, which circularises upon entry into the target cell nucleus to form an extrachromosomal DNA episome. The genome

contains 2-5 0.5kb tandem terminal repeats and 6-12 3kb tandem internal repeats, along with a short and long coding sequence containing almost one hundred genes expressed at different times during infection. Gene expression is controlled through expression of viral transcriptional transactivators and inhibitors, along with the use of several different promoters: W, C and Q are utilised during latent infection, and distinct promoters including F and Z are used during lytic infection (Chatila*, et al* 1997, Kieff and Rickinson 2007).

1.1.2 Infection with EBV

EBV is transmitted via salivary secretions from seropositive individuals, which infect the epithelial cells of the oropharyngeal cavity in a virus naïve target, possibly through initial adhesion onto the surface of resident B cells. Lytic infection of epithelial cells amplifies the number of infectious virions present, which subsequently infect naïve resting B cells residing in Waldeyer's ring (Cohen 2000, Thorley-Lawson and Allday 2008).

Following infection, transient expression of all latency proteins is observed (see section 1.1.3 below), resulting in activation and proliferation of the naïve B lymphocyte and migration to lymphoid follicles. Once the infected cell enters the germinal centre (GC), the pattern of gene expression switches to latency II, the default programme. Expression of this limited array of EBV genes provides the B cell with the survival signals necessary to undergo GC maturation, class switching and somatic hypermutation, thus mirroring the process driven by antigen exposure. The infected B cell enters the memory B cell compartment and EBV once again alters its gene expression profile to latency 0, whereby it evades the host immune response and can reside in the resting memory cell for the life of the individual (see Figure 1). Some controversy exists regarding EBV infection, for example whether EBV infects exclusively naïve or also mature B cells remains unclear, as does the location of EBV infected cells in either the GC or extrafollicular regions of tonsillar tissue (Kuppers 2003). However, the process described above is currently the generally accepted

model of EBV infection (Snow and Martinez 2007, Thorley-Lawson and Allday 2008).

Sporadic conversion to lytic infection can occur in a small proportion of infected B cells upon return to the oropharyngeal lymphoid tissue, or upon differentiation into a plasma cell. This results in re-infection of epithelial cells and release of virus in salivary secretions but is not normally associated with clinical symptoms and is controlled by the host immune response (Cohen 2000).

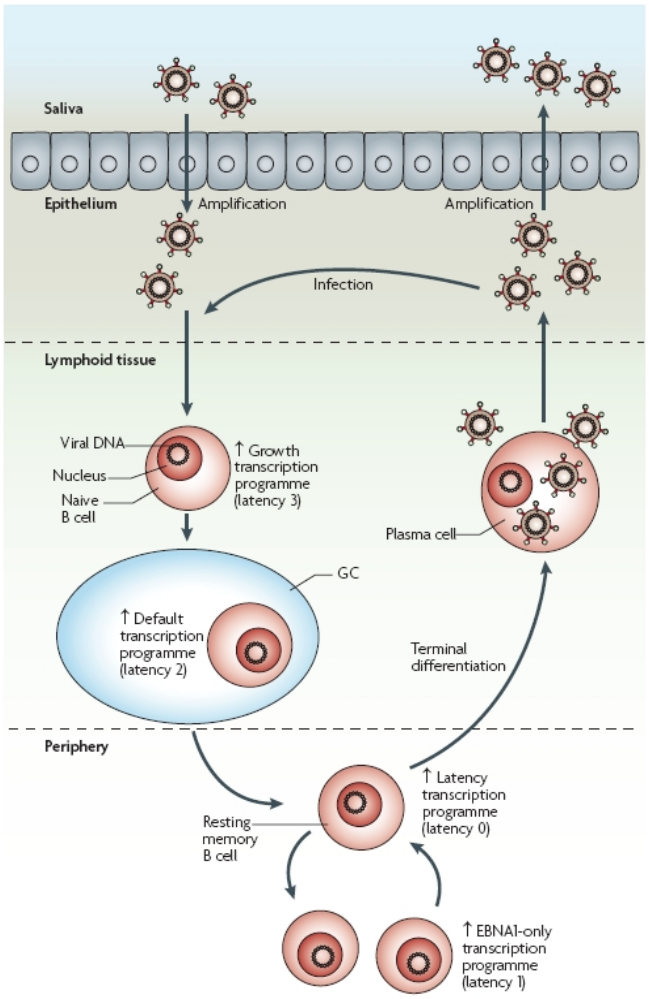


Figure 1: Illustration of EBV infection and persistence in the immune competent host. From Thorley-Lawson and Allday, 2008

Primary EBV infection generally occurs during childhood, often without clinical symptoms. However, in western society there is an increasing delay in primary exposure sometimes into the second or third decade of life. Primary infection during

adolescence or adulthood is associated with the development of infectious mononucleosis (I.M.) in an estimated 25% of cases. Symptoms of I.M. include the triad of pharyngitis, fever and lympadenopathy, which are though to be caused by the magnitude of the immune response to EBV rather than directly by the virus itself. These symptoms remain for a period of several weeks, with subsequent malaise that may persist for some months (Hislop*, et al* 2007).

1.1.3 EBV latency

EBV is capable of either lytic infection, resulting in production of infectious virus particles and death of the host cell, or latent infection where viral genes may be expressed but the virus is not propagated. Lytic infection is generally observed in epithelial cells or terminally differentiated plasma cells, whereas latent infection occurs in B lymphocytes. Four distinct programmes of viral latency have been identified under different circumstances in B lymphocytes *in vivo*, each expressing a selection of the 9 latency genes, but all expressing the non-coding RNAs (EBERs and BARTs) (see Table 1).

Latency 0, otherwise termed the "latency programme" is observed in resting memory B cells from healthy seropositive individuals, where no viral gene products are expressed when the cell is quiescent. Upon cell division as part of routine B cell homeostasis, EBNA-1 is transiently expressed to ensure replication of the EBV genome and distribution to daughter cells. Latency I is observed in Burkitt lymphoma cells, which express only EBNA-1. Latency II, the "default programme", has been identified in cells derived from patients with Nasopharyngeal Carcinoma or Hodgkin lymphoma, where EBNA-1, LMP-1 and LMP-2 are expressed. Finally, latency III, termed the "growth programme", is observed *in vivo* in most patients suffering from post transplant lymphoproliferative disease (PTLD). Latency III is also expressed *in vitro* by EBV-transformed B cell Lymphoblastoid Cell Lines (LCLs), and entails the expression of all 9 latency genes: EBNA-1, EBNA-2, EBNA-3a, 3b and 3c, EBNA-LP, LMP-1, LMP-2a and LMP-2b (see Table 1). Unless these cells are eliminated by the host immune response, expression of the full complement of latency proteins

results in B cell transformation and continued proliferation both *in vitro* and *in vivo* (Thorley-Lawson and Allday 2008).

Table 1: Patterns of latency in EBV infection. Detail of when each transcription program is observed both in health and disease is shown. EBNA: Epstein Barr Virus Nuclear Antigen. LMP: Latent Membrane Protein. EBER: Epstein Barr virus Encoded RNA. BART: BamA Rightward Transcripts. From Thorley-Lawson and Allday, 2008.

Transcription program	Cell type where found	Latent genes expressed
Growth (Latency 3)	Newly infected B cells Immunoblastic lymphoma	EBNA1 (Cp/Wp), EBNA2, EBNA3A, B, C, EBNALP, LMP1, LMP2A, B, EBER1, 2, BART
Default (Latency 2)	Germinal centre B cells Hodgkin lymphoma	EBNA1 (Qp), LMP1, LMP2A, B, EBER1, 2, BART
EBNA-1 only (Latency 1)	Dividing memory cells Burkitt Lymphoma	EBNA1 (Qp), EBER1 (?), 2 (?), BART(?)
Latency program (Latency 0)	Resting memory cells	EBER1, 2, BART (?)

The majority of PTLD tumours express the latency III EBV transcription profile, including all 9 latency proteins along with the EBERs and BARTs. The role of these genes has been the focus of extensive research to determine how EBV influences the growth of B and epithelial cells, the current understanding of which will be summarised below.

EBNA-1 is characterised by ubiquitous expression throughout all EBV latency profiles. It is essential for maintenance of the EBV episome during cell division, binding to the origin of replication OriP and associating with host cell chromosomes to ensure duplication of viral DNA, and equal distribution of episomes to daughter cells. EBNA-1 also has a role as a transcriptional transactivator, up-regulating viral protein

expression from the C promoter (Kieff and Rickinson 2007, Tanner and Alfieri 2001, Young and Murray 2003).

EBNA-2 has been described as a "master transactivator" and is absolutely required for transformation of B cells (Kieff and Rickinson 2007). An EBV strain deficient in EBNA-2 is unable to immortalise primary B cells in culture, however this ability returns with the restoration of EBNA-2 (Hammerschmidt and Sugden 1989, Rabson*, et al* 1982). EBNA-2 acts as a transcriptional activator for viral genes, including LMP-1, LMP-2 and also for the cellular activation markers CD21 and CD23, and anti-apoptotic genes including *c-myc* and *c-fgr*. The activity of EBNA-2 is potentiated 10-100 fold by the presence of EBNA-LP, which although not essential for transformation itself acts in parallel with EBNA-2.

EBNA-3 A, B and C have considerable sequence similarities, being produced from a single alternatively spliced primary transcript. EBNA-3A and C have been demonstrated as essential for transforming activity but EBNA-3B appears to be dispensable. These are also transcriptional regulators, being involved in both the down-regulation of EBNA-2 and the up-regulation of LMP-1 and cellular CD21.

LMP-1 is considered the major transforming protein of EBV and is a classical oncogene, the expression of which consistently induces tumour formation in mice. It resembles the surface molecule CD40, and acts as a constitutively active TNF receptor, signalling through both the MAP kinase and JAK/STAT pathways. This leads to stimulation of NFκB and cellular activation, resulting in the expression of cellular activation markers, cell surface adhesion molecules and cytokines. Anti-apoptotic molecules such as Bcl-2 and A20 are also upregulated by LMP-1 (Wang*, et al* 1990).

LMP-2A and B differ only in the absence of the intracellular domain from LMP-2B compared to LMP-2A, the most studied of the two to date. LMP-2A may substitute for BCR signalling in cells that have undergone crippling Ig gene mutations. Literature regarding the activity of LMP-2A suggests that it may have a dual role, being able to both block activation signals from the natural BCR, thus preventing activation-induced entry into the lytic cycle, and also mimic BCR signals in the absence of antigen. This

provides survival signals to cells which either cannot signal due to the lack of a functional BCR, or in the absence of antigen in the germinal centre, to drive passage into the memory B cell compartment. Some debate remains regarding the detection of LMP-2A, with some studies suggesting its expression in memory B cells from healthy individuals (Kuppers 2003). LMP-2B is thought to negatively regulate the activity of LMP-2A, therefore increasing the likelihood of a switch to lytic infection (Rechsteiner*, et al* 2008).

The EBERs and BARTs, despite ubiquitous expression in EBV infected cells, do not appear to be essential for transformation and their role remains to be elucidated. There is some evidence to suggest that the EBERs may mediate cellular responses to IFN-α signalling, however more work is required to establish the function of these RNAs.

The complement of latent EBV genes are therefore capable of providing the B cell with cellular activation signals, increased resistance to apoptosis, induction of autocrine cytokine secretion and prevention of entry into the lytic cycle. These signals appear to be sufficient to mimic B cell activation and therefore drive virus-initiated proliferation in the absence of normal cellular stimuli, which contributes to the development of neoplasia.

1.1.4 The immune response to EBV

Studies of the immune response to primary EBV infection have focussed on patients presenting with infectious mononucleosis, as these individuals are identifiably mounting such an immune response. The progression of this response has been studied in some detail, as has the memory response in healthy carriers. However, investigations of primary asymptomatic infection are not possible as these patients are unaware of seroconversion. It is therefore assumed that I.M. is an amplified representation of asymptomatic primary EBV infection. Although the immune response mounted towards EBV consists of both humoural and cellular components, the cellular arm predominantly controls viral infection.

In the acute phase of I.M., several anti-EBV antibodies are produced including IgM and IgG towards the Viral Capsid Antigen (VCA) and IgG antibodies towards the Early Antigen (EA). In addition to these, neutralising IgM and subsequently IgG antibodies appear that recognise the major glycoprotein gp350 and are capable of inducing antibody mediated cellular cytotoxicity. This complement of antibodies is unlikely to control established EBV infection, but may confer some protection against EBV re-activation, or re-infection with additional strains of EBV (Rickinson and Kieff 2007).

Aside from the magnitude of the CD8 response in I.M., the first and most compelling evidence that cellular immunity is critical in EBV control comes from the observation that T cell compromised patients are at increased risk of developing EBV-driven malignancies or viral lesions. In combination with the evidence that disease regression can be induced by infusion of EBV-specific T cells (Rooney*, et al* 1995), this indicates that CD8 positive EBV-CTLs are central for virus control (Rickinson*, et al* 1996).

During the acute phase of I.M., a five to 20 fold increase in the number of circulating CD8 T cells is induced. The CD8 cell count remains elevated throughout the acute phase and subsequently falls during convalescence (Rickinson and Kieff 2007). It has been demonstrated using tetramer analysis that during acute I.M. up to 50% of circulating CD8 T cells can be specific for a single EBV-derived epitope (Callan*, et al* 1998). Increased levels of CD4 T cells are also observed during I.M., of which up to 5% are EBV-specific, but this response is less marked than the observed CD8 cell expansion (see Figure 2). In addition, NK cell expansion has been identified in the peripheral blood of I.M. patients. This, along with the observation that NK cells are capable of inhibiting outgrowth of EBV-LCL *in vitro* by secretion of IFN-γ, may suggest a role for the innate immune system in delaying early progression whilst the adaptive response is mounted (Hislop*, et al* 2007).

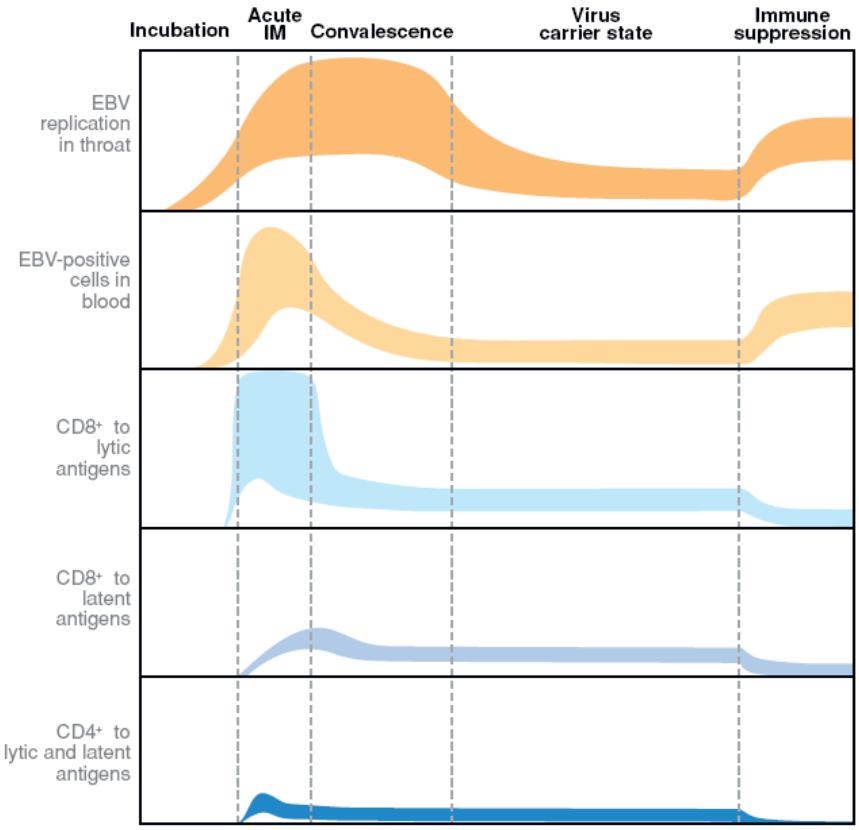


Figure 2: Illustration of the course of EBV infection. Representation of changes in EBV replication, number of circulating EBV infected cells and anti-EBV CD4 and CD8 cells. From Hislop, *et al* 2007.

It has been demonstrated that the CD8+ T cell response elicited during I.M. primarily recognises lytic EBV antigens, for which there is a strict hierarchy of immunological dominance. Most EBV-specific CD8 T cells recognise the immediate early proteins BZLF1 and BRLF1, with an additional subset directed towards early antigen proteins; but CD8 CTL responses rarely recognise the later expressed matrix proteins. This hierarchy closely reflects the efficiency of antigen processing and presentation by MHC class I molecules (Pudney, *et al* 2005). EBV-CTLs recognising latent proteins are also detected during acute I.M., but at a comparatively low frequency. However, during recovery from acute infection the number EBV-specific CTLs falls to represent 2-5% of total CD8 T cells, with a disproportionately greater reduction in lytic antigen specific cells such that the majority of remaining EBV-CTLs in healthy carriers recognise latent rather than lytic proteins.

The composition of this CD8 response towards latent antigens conforms to a different hierarchy of EBV proteins, being comprised mainly of EBNA-3 (A, B or C) specific cells, with a small proportion of anti-LMP-2 cells detected. This protein hierarchy is conserved across many HLA types, with differences in the epitope specificity between alleles (Rickinson*, et al* 1996). The mechanisms for immunodominance of the EBNA-3s and LMP-2 have not been elucidated, although the EBNA-3s comprise approximately 60% of the unique sequences present in EBV latent proteins and therefore could be expected to represent many of the recognised epitopes (Hislop*, et al* 2007). Early work identified the presence of a Glycine-Alanine repeat (GAr) within the EBNA-1 protein, which was suggested to inhibit proteosome processing and therefore preclude presentation to CD8+ T cells in MHC class I (Levitskaya*, et al* 1995). However, EBNA-1 reactive CD8 clones have since been identified (Voo*, et al* 2004), and it is possible that the GAr merely prevents translation from EBNA-1 mRNA, rather than actively interrupting proteosomal processing (Lee*, et al* 2004, Tellam*, et al* 2004, Yin*, et al* 2003).

In contrast, CD4 responses elicited by EBV infection adhere to a different profile of immunodominance. During IM, responses to the lytic proteins BZLF1 and BHLF1 as well as the late glycoproteins gp350 and gp110 have been documented. Although CD4 memory cells following recovery are present at a ten-fold lower frequency than CD8 memory cells, CD4 responses can be reactivated *in vitro*. Unlike CD8 cells, which are mainly EBNA-3 specific, CD4 cells are commonly directed against EBNA-1, EBNA-2 and EBNA-3C with much lower frequencies detected against EBNA-3A or the LMPs. These CD4 responses tend to have a Th1 phenotype, with the ability to secrete IFN-γ and are often cytotoxic. Therefore it is possible that these EBV-specific CD4+ cells in fact also play the role of cytotoxic effectors rather than fit the traditional profile of helper cells (Rickinson and Kieff 2007).

Generation of an effective immune response to EBV infection is critical in control of the virus. This is illustrated by the clinical course observed in X-linked lymphoproliferative disease (XLP) patients. These patients have a defect in cellular immunity caused by the absence or non-functionality of the signalling protein SAP (SLAM-associated protein), resulting in reduced cytotoxic responses and inability to

control EBV infection. As a result, XLP patients can develop fulminant I.M. and EBV driven lymphoma (Nagy*, et al* 2009). This illustrates the potential for EBV to cause disease if an ineffective immune response is generated.

1.1.5 Association of EBV with malignancy

Along with its ability to transform primary B cells into continuously proliferating LCL lines in culture, EBV's status as an oncovirus is confirmed by its association with several human malignancies. These tumours reflect the natural tropism of EBV for B lymphocytes and epithelial cells, and include Burkitt Lymphoma, Post Transplant Lymphoma, Hodgkin lymphoma, Nasopharyngeal carcinoma, a subset of gastric carcinomas and some rare T/NK cell lymphomas. The role of EBV in the development of some of these tumours is not clearly defined despite a large body of work examining its contribution as an associated, predisposing or causative factor.

EBV was first identified from African Burkitt lymphoma (BL) cell lines, initially seeming to play a causal role following identification in almost all samples tested expressing the latency I programme. However, it has since become clear that EBV is merely one of many factors contributing to the development of this disease which include both immune exhaustion as a result of malaria infection as well as the diagnostic characteristic of BL, the *c-myc* translocation. It is thought that the translocation of *c-myc* to place it under the influence of the constitutively active Ig promoters drives growth, and that the presence of EBV may allow escape from apoptosis (Thorley-Lawson and Allday 2008).

EBV is detected in up to 50% of Hodgkin Lymphoma (HL) samples, with abundant LMP-1 expression in Reed-Sternberg cells (Young and Murray 2003). Furthermore, EBV-positive HL patients have higher titres of anti-EBV antibodies compared to other lymphoma patients (Levine*, et al* 1971). There appears to be underlying differences in the aetiology and behaviour of EBV positive/EBV negative HL however the exact mechanism by which EBV contributes to lymphomagenesis remains to be elucidated. Work by Mancao *et al* identified a critical role for LMP-2A signalling in promoting

the survival of B cells carrying a crippled Ig gene rearrangement. This finding is significant as approximately 25% of HD Reed-Sternberg cells do not express a functional B cell receptor due to Ig mutations, and would be expected to undergo apoptosis. Therefore the action of LMP-2A provides a role for EBV in the development of HD (Mancao*, et al* 2005, Mancao and Hammerschmidt 2007).

Undifferentiated nasopharyngeal carcinoma (NPC) is uniformly associated with EBV, with EBERs and EBNA-1 expressed in most tumour samples. In addition, LMP-1 is expressed in a large proportion of tumours (Young and Murray 2003). EBV is found in epithelial cells comprising the lesions, but not the associated lymphocytes. Patients with undifferentiated NPC often have raised titres of IgA antibodies that recognise EBV structural proteins, which can be associated with poorer prognosis (Cohen 2000). Once again, the contribution of EBV to disease development is unclear, and other factors, eg. diet, may also be important.

It can be concluded that EBV is likely to play a role in the development of several tumours worldwide, however the mechanism of this contribution is elusive, and is likely to differ for each circumstance. One possibility is that EBV contributes according to a "first hit" model, whereby the presence of virus predisposes to tumorigenesis but EBV is not involved in maintenance or progression of the disease. The role of EBV in the development of post transplant lymphoma is more clearly defined, and will be discussed below.

1.2 Post Transplant Lymphoproliferative disease

PTLD is a significant complication of both stem cell and solid organ transplantation, and is associated with considerable morbidity and mortality. As transplantation medicine progresses, transplantation has become an increasingly possible therapeutic option and patients survive longer following the procedure, therefore the impact of PTLD has increased (Everly*, et al* 2007). Currently, PTLD is the most common post transplant malignancy in paediatric patients, and is second only to skin cancers in adult patients, affecting between 1 and 35% of transplanted individuals (Abu-Elmagd*, et al*

1998, Opelz and Dohler 2004, Taylor, *et al* 2005, Webber, *et al* 2006). Five year mortality associated with the disease can reach 50% despite current treatment regimens (Caillard, *et al* 2005, Leblond and Choquet 2004). PTLD remains a considerable problem following transplantation and more effective treatment options are required.

1.2.1 Clinical presentation and classification of PTLD

Presentation of PTLD can be variable, therefore a high index of suspicion is required in all patients post transplant, however up to 80% present with fever, 50% with lymphadenopathy, and non-specific symptoms including malaise and weight loss are also commonly seen. Tonsillar involvement or symptoms reflective of I.M. are frequent (Everly, *et al* 2007, Taylor, *et al* 2005), and extranodal presentation such as small bowel bleeding or CNS involvement may also be seen, particularly in T cell PTLD. A minority of patients (approximately 10%) have bone marrow involvement, and occasional patients may be asymptomatic.

The term PTLD encompasses a heterogeneous group of B cell proliferative disorders that range from polymorphic B cell hyperplasia to aggressive, high grade, monoclonal B cell lymphoma. The WHO classification of PTLD recognises three distinct categories (Harris, *et al* 2000). Early lesions or hyperplastic PTLD are characterised by non-invasiveness, with preservation of local tissue structure and often affect tonsils or lymph nodes. Early PTLD is associated with a good prognosis and is likely to respond to a reduction of immunosuppressive therapy. Polymorphic PTLD begins to invade local tissue architecture and can involve nodal and extranodal sites. Areas of necrosis are often observed along with atypical nuclear structure of involved B cells. Polymorphic PTLD is the most common stage, and is less likely to respond to a reduction of immunosuppression alone, requiring further interventions. Finally, Monomorphic PTLD often resembles Non-Hodgkin Lymphoma, is high-grade and invasive with destruction of local tissue architecture and involvement of extra-nodal sites (Lim, *et al* 2006, Taylor, *et al* 2005). Both polymorphic and monomorphic PTLD are generally of B cell lineage but approximately 5-10% of patients show T cell

lymphoproliferation. Rare "other types" of post transplant lymphoma represent a fourth category, see Table 2. It should be noted however that data regarding the clinical significance of histological presentation are conflicting (Hayashi, *et al* 2001, Pinkerton, *et al* 2002).

Table 2: WHO classification of PTLD. Three main categories are recognised based mainly on morphological features. From Harris et al, report from the WHO meeting, 2000.

1. Early lesions Reactive plasmacytic hyperplasia Infectious mononucleosis-like
2. PTLD – polymorphic Polyclonal (rare) Monoclonal
3. PTLD monomorphic (classify according to lymphoma classification) • B-cell lymphomas Diffuse large B-cell lymphoma (immunoblastic, centroblastic, anaplastic) Burkitt/Burkitt-like lymphoma Plasma cell myeloma • T-cell lymphomas Peripheral T-cell lymphoma, not otherwise categorized Other types (hepatosplenic, gamma-delta, T/NK)
4. Other types (rare) Hodgkin's disease-like lesions (associated with methotrexate therapy) Plasmacytoma-like lesions

1.2.3 PTLD risk factors

A multitude of analyses assessing PTLD have confirmed that it arises in between 1 and 35% of solid organ transplant recipients (Abu-Elmagd, *et al* 1998, Abu-Elmagd, *et al* 2009, Caillard, *et al* 2005, Cockfield 2001, Issa, *et al* 2009, Knight, *et al* 2009, Opelz and Dohler 2004, Shiba, *et al* 2004, Webber, *et al* 2006). This range of incidences can be further subdivided into risk based on whether the patient is adult or paediatric, the nature and intensity of immunosuppression used, and the type of organ transplanted (Knight, *et al* 2009, Opelz and Dohler 2004).

The increased risk identified for paediatric compared to adult patients has been attributed not to difference in recipient age per se, but to the fact that the majority of

adults are seropositive at the time of transplant, whereas a large proportion of children are EBV naïve and experiencing primary infection in an immunosuppressed state (Caillard, *et al* 2005). Primary EBV infection occurring in an individual unable to mount a cellular immune response results in very high rates of PTLD development, with risk increased by 10-76 fold (Preiksaitis 2004). EBV seronegativity prior to transplantation followed by subsequent EBV infection is well established as a major risk factor for PTLD development (Ho, *et al* 1985, Shahinian, *et al* 2003, Walker, *et al* 1995), see Figure 3.

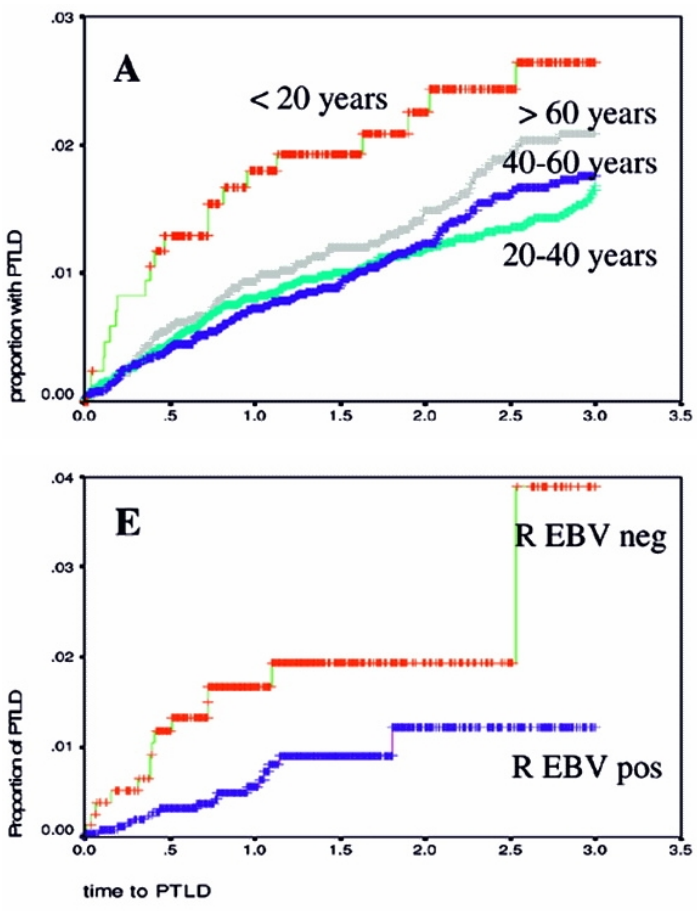


Figure 3: Increased risk of PTLD in patients under 20 years of age and EBV negative recipients. The proportion of patients aged <20 years developing PTLD is increased compared to all other age groups. EBV seronegative recipients also have increased risk of PTLD. From Caillard *et al*, 2005.

In addition, PTLD risk is strongly associated with the type of organ grafted. It is likely that this is reflective of the differing immunosuppression requirement for different organs. Immunostimulatory grafts, such as intestinal or heart-lung transplants require stronger immunosuppression to prevent rejection compared to those that provoke less intense allogeneic responses and therefore require lower immunosuppressive doses eg kidney transplants (see Table 3).

Table 3: Incidence of PTLD by transplant type. Adapted from (Cockfield 2001).

Transplant type	Incidence of PTLD
Kidney	1%
Liver	2.2%
Heart	3.4%
Lung	1.8-7.9%
Heart-Lung	9.4%
Intestinal	7-11%
Multivisceral	13-33%
Bone Marrow	<1%

Some studies have found that certain immunosuppressive agents are associated with higher risk of PTLD development (Opelz and Dohler 2004, Penn 1987), however this has not been observed in other studies (Birkeland and Hamilton-Dutoit 2003, Landewe*, et al* 1999). It seems likely that PTLD risk is increased by a higher cumulative dose of immunosuppression, rather than by a particular agent (Brumbaugh*, et al* 1985, Sokal*, et al* 1997). However, one US study of over 25000 renal transplant recipients suggested an increased risk of PTLD in patients receiving FK506 compared to CsA, a finding supported by the study of Opelz in 2004. In addition, patients treated with Mycophenolate Mofetil (MMF) may have a reduced risk of PTLD, possibly because MMF also has a suppressive effect on B cells (Caillard*, et al* 2005). The role of individual IS agents is therefore not entirely clear, however in general highly immunosuppressive regimens are associated with increased PTLD compared with less

immunosuppressive regimens (Caillard*, et al* 2005, Gutierrez-Dalmau and Campistol 2007).

Despite the conclusion that PTLD is the result of immunosuppression generally rather than a specific agent, it should be noted that the use of T cell depletion (TCD) in both stem cell and solid organ transplantation is associated with increased risk of PTLD development. T cell depletion is an effective strategy for graft versus host disease (GVHD) prophylaxis either *in vivo* using anti-thymocyte globulin (ATG) or of the graft itself pre-infusion, however these are also major PTLD risk factors in the SCT setting. Indeed, PTLD is rarely observed in SCT in the absence of *in vitro* or *in vivo* T cell depleting antibodies (Caillard*, et al* 2005, Landgren*, et al* 2009, Lynch*, et al* 2003). This risk can be reduced by depleting both T and B cells from the stem cell graft rather than T cells alone (Liu*, et al* 2004). Various T cell depleting strategies are also used as both induction therapy and for treatment of rejection episodes in SOT, however many studies have found that TCD is a risk factor for PTLD development in this setting also (Issa and Fishman 2009).

It has been suggested that the grafted organ itself contributes to the pathogenesis of PTLD via chronic low-level antigen stimulation, a hypothesis that is supported by the relatively high incidence of PTLD involving the transplant (Birkeland 1983, Lim*, et al* 2002). However, it is difficult to separate the relative contributions of increased immunostimulation by a particular type of graft from the heavier immunosuppression required to protect it from rejection. Increased lymphoma risk is also seen in patients with inherited or acquired immunodeficiencies (eg. Wiskott-Aldrich Syndrome, HIV infection), or following immunosuppressive treatment for autoimmune conditions, suggesting that immune compromise itself is the main risk factor rather than organ grafting (Birkeland and Hamilton-Dutoit 2003).

1.2.4 The role of EBV in PTLD development

The relationship between iatrogenic immunosuppression in organ transplant recipients and an increased risk of lymphoma has long been recognised, and the association with

EBV infection is now well established (Buell, *et al* 2005, Caillard, *et al* 2005, Ho, *et al* 1985, Rickinson, *et al* 1996, Shahinian, *et al* 2003, Swerdlow, *et al* 2000, Walker, *et al* 1995). Over 90% of PTLD lesions are EBV positive, including over 90% of B-lineage and approximately 50% of the much rarer T-lineage tumours. There are several lines of evidence suggesting that EBV plays a role in the development of these tumours. First, EBV can be detected in over 90% of tumour tissue, and EBV associated RNAs (EBERs) frequently co-localise to the malignant cells histologically. Furthermore, in these tumour samples EBV genomes are often clonal, indicating that the virus was present at the time of transformation. The EBV viral load in peripheral blood samples often rises prior to development of PTLD, particularly in SCT patients. In addition, the well characterised oncogenic potential of the expressed EBV genes *in vitro* demonstrates that EBV has the capacity to drive the development of PTLD. The role of EBV is further highlighted by tumour regression which can be induced by the infusion of EBV-specific T cells (Rooney, *et al* 1998). Therefore, the weight of evidence strongly suggests that EBV is centrally involved in the pathogenesis of PTLD (Nalesnik 1998, Tsao and Hsi 2007).

As discussed above, in the immune competent host EBV infection is controlled by the cellular immune response. Infected cells normally express the latency 0 programme, with efficient recognition and removal of any that express immunogenic EBV derived proteins. However, upon compromise of cellular immunity, the immune environment becomes permissive for EBV infected cells to establish other latency phenotypes, escape recognition or elimination by CTLs and drive B cell proliferation. This is observed in several groups of immunocompromised patients: in stem cell transplant patients prior to reconstitution of T cell responses from the graft, during pharmacological immunosuppression to prevent graft rejection following solid organ transplant, in immunodeficient HIV patients, or in patients with primary immunodeficiencies such as Wiskott-Aldrich syndrome and XLP (Hopwood and Crawford 2000, Young and Murray 2003). Therefore the critical factor in control of EBV infection is intact EBV-specific cellular immunity, which maintains the virus is its quiescent state without clinical symptoms.

However, despite these observations regarding the role of EBV in the development of PTLD, it is clear that the combination of immunosuppression and EBV infection alone is not sufficient for tumour formation. The majority of transplanted patients receive immunosuppressive therapy for many years without development of PTLD. It is thought that several other factors may allow EBV to drive outgrowth of B cells, including the cytokine environment (eg. TNFα mutations, IFNγ levels) (Lee, *et al* 2006b, McAulay, *et al* 2009) and the accumulation of additional genetic changes in proliferating B cells (eg. *bcl-6* and *c-myc* mutations) (Lim, *et al* 2006).

It should also be noted that a subset of PTLD consisting of up to 10% of cases is EBV-negative (Leblond, *et al* 1998). This well recognised but poorly understood group of malignancies are more aggressive, tend to occur late after transplantation and can include either B or T cell lymphomas (Ghobrial, *et al* 2005, Nelson, *et al* 2000, van Gorp, *et al* 1994). It has been suggested that they should be considered as a separate entity from early, EBV positive PTLD as their clinical behaviour more closely reflects spontaneous lymphoma in the immunocompetent population and a distinct gene expression profile has been identified in these tumours (Craig, *et al* 2007).

1.2.5 Prediction of PTLD

Despite the recognition of several factors that increase PTLD incidence in various cohorts of patients, identification of the individual patient likely to develop the disease remains a challenge.

PTLD prediction differs considerably between stem cell and solid organ transplant patients. In patients undergoing T cell depleted SCT, EBV viraemia in the first few months post transplant appears predictive of PTLD, which enables pre-emptive intervention allowing good results in several studies (Ahmad, *et al* 2009, Gustafsson, *et al* 2000, Omar, *et al* 2009, Wagner, *et al* 2004). However, while PTLD very rarely occurs in the absence of EBV viraemia, the positive predictive value of viraemia is variable, the optimal threshold for intervention in these patients has yet to be determined, therefore patients in some of these trials were probably over-treated

(Greenfield, *et al* 2006). Meij *et al* have demonstrated that combining measurement of EBV viral load with the presence or absence of EBV-specific CD8 T cells as assessed by HLA-peptide tetramers increased the positive predictive value of EBV viraemia from 30% to 100% in patients undergoing T cell depleted SCT (Meij, *et al* 2003), but these assays are not yet in routine clinical use. Current practice generally includes close monitoring of viral load in such patients, with pre-emptive treatment in patients at highest risk.

In SOT patients the association between increased EBV load and development of PTLD is less clear, as many patients with high viral loads do not progress to development of PTLD (Benden, *et al* 2005, Doesch, *et al* 2008). Attempts to predict those patients most likely to develop PTLD have utilised several assays examining cytokine polymorphisms (Lee, *et al* 2006b), general measures of cellular immunity (Lee, *et al* 2006a) or CD4/CD8 counts (Sebelin-Wulf, *et al* 2007) in combination with EBV viraemia. The most successful of these combined the two contributing factors in PTLD development: EBV load and anti-EBV immunity. Smets *et al* measured EBV copy number per μg DNA in conjunction with EBV specific immunity using ELISPOT, resulting in the PTLD index (I_{PTLD}) with 86% sensitivity, 100% specificity and positive predictive value, and 92% negative predictive value (Smets, *et al* 2002). This represents a significant improvement on previous attempts to predict PTLD which often had poor positive predictive value; however the use of I_{PTLD} is limited by the requirement to establish an LCL line from each patient to measure EBV specific immunity. Should a more rapid and reliable method for measurement of functional EBV-immunity become available, the I_{PTLD} index promises to be useful in predicting the highest risk patients. Alternatively, combination of EBV load with more general measures of immune capability in immunosuppressed patients may prove to be an adequate predictive measure. The ability to identify those patients at highest risk would be beneficial for pre-emptive treatment of PTLD, allowing earlier intervention and the initiation of patient-specific therapies. Early application of these approaches could reduce PTLD related mortality.

1.3 Current treatment strategies for PTLD

Strategies to treat PTLD are currently based on three main approaches: restoration of anti-EBV immunity, reduction of tumour burden, or the elimination of EBV itself (Svoboda*, et al* 2006) (see Figure 4).

1.2.1 Reduction of immunosuppression

First line treatment generally addresses the former issue, with reduction of immunosuppressive therapy aiming to allow reconstitution of the cellular immune response to EBV and subsequent control of proliferating, EBV infected B cells. Reducing the level of immunosuppression alone provokes a clinical response in up to 60% of patients, especially in those who are diagnosed early and are suffering from early lesions or polymorphic PTLD (Tsai*, et al* 2001). The efficacy of this approach was illustrated by Guppy *et al*, with demonstration of increased EBV-specific immunity concurrent with reduced IS in nine patients (Guppy*, et al* 2007). However, this approach carries a considerable risk of either transplant rejection in the SOT patient, or development of GVHD in the SCT patient. In fact, in one major series examining outcome following heart transplantation, death from graft loss following reduction in immunosuppression was as common as death from PTLD (Webber*, et al* 2006).

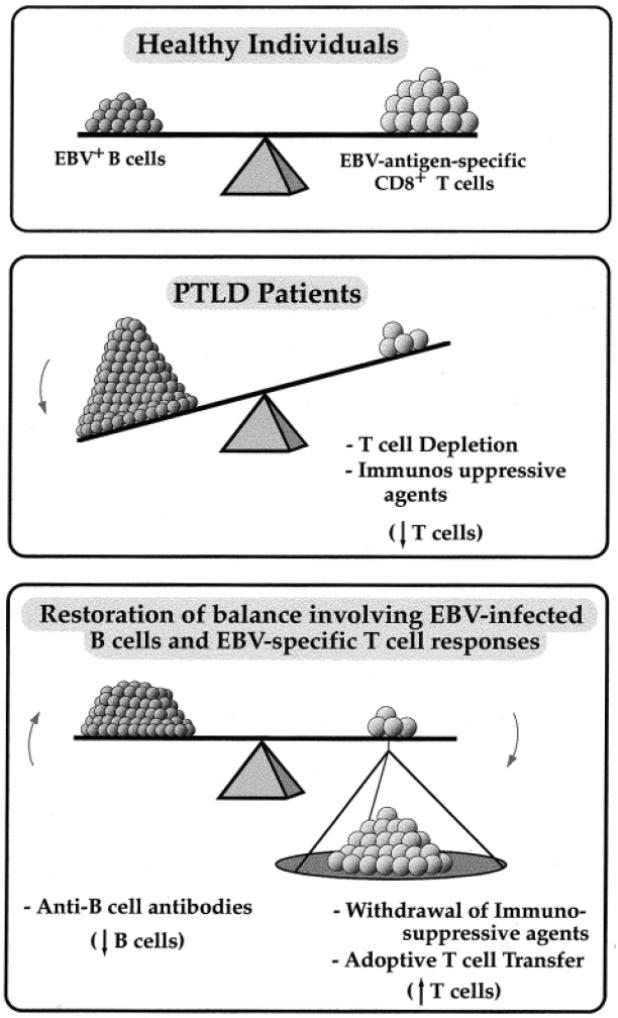


Figure 4: Illustration of balance between EBV infected B cells and EBV-specific T cells. Top panel: Maintenance of balance in healthy individuals ensures control of EBV infection. Middle panel: EBV-infected B cells proliferate in immunosuppressed patients, leading to PTLD. Lower panel: Restoration of balance by reduction of B cells or augmentation of T cell responses. (Hsieh*, et al* 1999)

1.2.2 Monoclonal antibodies

In order to reduce tumour bulk, B cell specific monoclonal antibodies were first employed against PTLD in 1991, with the use of anti-CD21 and CD24 inducing complete remission in 16 of 26 treated patients (Fischer*, et al* 1991). These antibodies were subsequently replaced by the chimaeric anti-CD20 monoclonal antibody Rituximab, which was introduced in 1998 and has since become widely used. The

target molecule CD20 is expressed in approximately 90% of PTLD tumours, and binding of Rituximab to this surface marker results elimination of the cell. Several mechanism are thought to be involved in this process, including complement mediated cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC) and apoptosis (Zhou*, et al* 2008). In addition, the presence of NK cells has been demonstrated to be important in Rituximab action in one study (Markasz*, et al* 2009).

As outlined above, Rituximab appears effective as pre-emptive treatment in patients undergoing T cell depleted SCT who develop EBV viraemia. Several trials have addressed the efficacy of this drug when used as treatment for established PTLD, with response rates of 44-75% when Rituximab is used as single agent therapy, with complete remission achieved in 28-75% (Choquet*, et al* 2006, Svoboda*, et al* 2006). However, more recent data have suggested that Rituximab therapy is associated with significant rates of partial response and relapse, likely due to the transient depletion of B cells achieved (Choquet*, et al* 2007, Messahel*, et al* 2006). In addition, it has been suggested that the pan-B cell depletion resulting from treatment with Rituximab could compound the already profound immunodeficiency experienced by patients post-transplant (Verschuuren*, et al* 2002), although the impact of this on antibody levels and infections has been disputed by other authors (Davis*, et al* 1999).

1.2.3 Chemotherapy

Chemotherapy is a widely used and effective treatment for non-PTLD malignancies in the general population, and is also used as therapy for EBV-driven lymphoma in transplant patients. Low dose chemotherapy with cyclophosphamide and prednisolone appears to be an effective therapy for PTLD in the SOT setting (Gross*, et al* 2005). Despite response rates of up to 70%, toxicity is a serious consideration, with treatment related mortality of 25% (Elstrom*, et al* 2005, Svoboda*, et al* 2006). Furthermore, in SCT patients, prolonged courses of even low dose chemotherapy may have a deleterious effect on the stem cell graft. Currently, a prospective, randomised study is underway in paediatric PTLD comparing cyclophosphamide/prednisolone chemotherapy alone or in combination with Rituximab.

1.2.4 Other approaches

For patients with localised, accessible disease, surgical resection combined with reduction of immunosuppression may be highly effective (Green 2001). Administration of anti-viral agents such as ganciclovir to combat EBV proliferation remains controversial for PTLD treatment. While two studies suggested that ganciclovir treatment in the immediate post transplant period can reduce the development of PTLD in kidney, liver and pancreas transplant recipients (Darenkov*, et al* 1997, McDiarmid*, et al* 1998), other studies show no effect of anti-viral therapy (Davis 1998). Due to their mode of action targeting the lytic cycle of EBV, it is unlikely that these drugs would have an impact on latently infected proliferating B cells (Crumpacker 1996). However it remains possible that release of infectious virus from lytically infected cells compounds the situation in PTLD patients and interruption of this pathway could be beneficial. As with the other therapies above, anti-viral drugs have their own toxicity profile, including myelosuppression by ganciclovir and nephrotoxicity with cidofovir.

In conclusion, all treatment options that are routinely available for PTLD at present are associated with negative effects: reduced IS increases the risk of rejection, Rituximab may compound immunosuppression and has significant relapse rates, chemotherapy carries considerable treatment related toxicity and the efficacy of anti-virals is not established. The drawbacks of these currently available therapeutic options has led to the development of new approaches to PTLD treatment. Intuitively, the most logical approach to this is to correct the underlying deficit in EBV-specific immunity.

1.4 Adoptive immunotherapy

The term immunotherapy refers to the use of immunological agents to treat disease. In the case of PTLD, there is an underlying deficiency in the cellular immune response, which allows EBV to drive inappropriate B cell growth. PTLD is an ideal candidate for the application of immunotherapy due to expression of EBV latent proteins on tumour cells, which therefore constitute an immunogenic target. One approach to

immunotherapy for EBV-PTLD is to correct the underlying deficit in cell mediated immunity by administration of EBV-specific cytotoxic T cells, generated *ex vivo*, which home to the tumour and lyse EBV-expressing cells.

As immunotherapy in this setting targets the cause of the disease rather than treating the resulting tumour, it has several advantages over conventional therapies; it is specific to the tumour cells therefore causing fewer side-effects, and is long-lasting as the EBV-CTLs establish immunological memory and persist to control disease long term. However, a major drawback of immunotherapy currently limiting its more widespread use is the requirement for generating a new cell line for each patient, requiring considerable time, resources and technical expertise.

1.4.1 Immunotherapy in stem cell transplantation

The potential for immunotherapy to reconstitute anti-viral immunity post stem cell transplantation was first established in 1992 with infusion of T cell clones to three patients which successfully controlled CMV disease following stem cell transplant (Riddell*, et al* 1992). This preliminary study was followed by a larger series in which CMV specific CTLs were infused pre-emptively into patients developing CMV viraemia post SCT. CTLs were well tolerated and no CMV viraemia or disease were observed in 14 patients (Walter*, et al* 1995). Since these initial studies, adoptive immunotherapy for CMV has been used successfully in over 50 patients (Einsele*, et al* 2002, Peggs*, et al* 2003).

Immunotherapy for EBV-PTLD arising after stem cell transplantation was also mush studied during this period. Initially, infusion of unmanipulated donor lymphocytes (DLI) was explored as a means to boost T cell responses prior to reconstitution of cellular immunity from the stem cell graft. Despite inducing regression of established PTLD in 6 patients, the presence of alloreactive T cells within the DLI led to an unacceptably high incidence of GVHD as a result of this therapy (Heslop*, et al* 1994b, Papadopoulos*, et al* 1994).

Therefore, although total lymphocyte preparations from the stem cell donor can be effective against PTLD, removal of the alloreactive cells and concomitant enhancement of the EBV compartment was desirable to circumvent the initiation of GVHD. This was achieved by multiple stimulations of donor peripheral blood mononuclear cells (PBMCs) with autologous EBV-transformed B cell lines *in vitro*. This technique results in expansion and activation of EBV-specific cells and loss of alloreactivity, generating EBV-specific, MHC-restricted cytotoxic T cell lines. Infusion of donor-derived EBV-specific CTL lines into 10 patients post stem cell transplant was well tolerated, with no *de novo* EBV disease developing, control of active EBV replication in two patients and resolution of frank lymphoma in one (Heslop*, et al* 1994a, Rooney*, et al* 1995). This study confirmed that EBV-CTLs can prevent and control EBV-driven disease, do not cause GVHD, and importantly that the infused cells can persist for some time after administration. This was the first successful trial of adoptive immunotherapy for PTLD and paved the way for further studies.

In subsequent studies, it was established that adoptively transferred CTLs persist long-term *in vivo* through retroviral gene marking of infused CTLs with the Neomycin resistance gene (Heslop*, et al* 2009). In addition, infusion of EBV-specific CTLs to patients at high risk of PTLD after T cell depleted SCT effectively prevented PTLD when compared to a historical control cohort (Rooney*, et al* 1998). To date, EBV-CTLs have been administered to over 200 patients at multiple different institutions (Gustafsson*, et al* 2000, Hale*, et al* 2008, Merlo*, et al* 2008). These studies have established adoptive immunotherapy with EBV-specific CTLs to be safe and effective in both preventing and treating EBV-PTLD after T cell depleted stem cell transplant. Therefore in this setting, reconstitution of the EBV compartment of the immune system can be achieved using adoptive transfer of *ex-vivo* expanded donor EBV-CTLs, which effectively controls PTLD.

1.4.2 Immunotherapy in solid organ transplantation

Following the success of adoptive immunotherapy in the setting of stem cell transplantation, this approach has also been applied to PTLD arising after solid organ transplant. In contrast to SCT patients, PTLD arising after SOT is almost exclusively of recipient origin and therefore recipient EBV-CTLs, or EBV-CTLs which are HLA-matched to the recipient are required. Immunotherapy trials post SOT can be divided into those utilising patient derived (autologous) CTLs and those using third party derived, HLA-matched donor CTLs (allogeneic).

1.4.2.1 Autologous EBV-CTLs

Immunologically, autologous EBV-CTLs are the ideal product to combat recipient derived EBV reactivation in the SOT patient. Expression of EBV latent antigens in LCLs, which are used as APCs for CTL generation *in vitro,* is identical to that observed in most PTLD tumours. Therefore, EBV-CTLs produced by exposure to these LCLs are a polyclonal cell line targeting the tumour's immunogenic antigens in the context of the correct HLA alleles.

Adoptive immunotherapy with autologous EBV-CTLs generated from patient blood taken pre-transplant was first administered to three SOT patients in 1998, resulting in transient increases in CTLp frequency and reductions in EBV viraemia. This study established the feasibility and safety of CTL infusion in the SOT setting (Haque*, et al* 1998). It was subsequently established that EBV-CTL lines can be reactivated from patients receiving immunosuppressive therapy with identical growth kinetics to those derived from healthy donors (Khanna*, et al* 1999, Savoldo*, et al* 2001). In addition, one autologous CTL line generated by Khanna *et al* was administered to a patient with progressive PTLD. Complete remission was induced, thus demonstrating the potential for clinical efficacy in SOT patients. However, secondary lesions developed in this patient, raising questions regarding the persistence of EBV-CTLs in the presence of immunosuppression and their ability to control disease long-term (Khanna*, et al* 1999).

Subsequently, the effect of autologous EBV-CTLs in SOT patients has been assessed in four main studies performed between 2002 and 2006, treating 25 patients either prophylactically or for established disease. Immunological parameters and clinical responses in these patients consistently suggest that, while adoptive immunotherapy can induce responses, these can be partial or temporary (Comoli*, et al* 2002, Comoli*, et al* 2005, Savoldo*, et al* 2006, Sherritt*, et al* 2003). Categorisation of patients treated in all studies thus far according to prophylaxis or treatment shows that, of 39 evaluable patients treated for established PTLD, 19 achieved complete remission, 3 partial remissions were observed and 16 did not respond. One patient has stable disease. Of 21 patients at risk of PTLD treated prophylactically, no PTLD was observed (Merlo*, et al* 2008).

Expansion and persistence of the infused CTLs post SOT is generally modest compared to that observed following HSCT, however some responses are achieved. Several trials have shown an increase in EBV-CTLp frequency following infusion of autologous EBV-CTLs, for example in one study increases of 1.5-4.8 fold were documented (Savoldo*, et al* 2006). However, it was generally noted that these increases were transient, often dropping again between cell infusions, sometimes to pre-treatment levels (Comoli*, et al* 2002, Haque*, et al* 1998, Savoldo*, et al* 2006). It is possible that in the presence of immunosuppressive therapy, the proliferative potential of infused EBV-CTLs is curbed resulting in limited *in vivo* expansion and persistence following administration. However, infusion of EBV-CTLs has been shown to have an impact on EBV viraemia in some patients, with reductions in EBV-load documented. Although these are also sometimes transient decreases, some patients experience sustained lowering of EBV genomes and therefore reduced risk of PTLD development (Comoli*, et al* 2002, Haque*, et al* 1998). In contrast, one study demonstrated an increase in plasma EBV-DNA consistent with lysis of infected cells approximately 2 weeks following EBV-CTL infusion; however this was not accompanied by a subsequent reduction in EBV-DNA detected in PBMC samples (Savoldo*, et al* 2006).

The body of evidence thus far illustrates that EBV-CTL therapy has considerable promise in the setting of post-SOT PTLD, with clinical responses induced in some patients. However, for many patients the infusion of EBV-CTLs is an adjunctive

therapy alongside or following several conventional approaches, therefore attributing these responses to immunotherapy alone can be difficult. In conclusion, whilst promising, the use of EBV-CTLs in the setting of SOT remains to be fulfilled to its potential.

1.4.2.3 Generation of EBV-CTLs from seronegative recipients

As discussed above, EBV negativity at the time of transplant is a major risk factor for PTLD in SOT recipients, and many paediatric patients fall into this category. However, current protocols that are routinely used for generation of EBV-CTLs from PBMCs are based on re-activation of memory CTLs within the T cell compartment. EBV negative recipients do not have memory EBV-CTLs, therefore cell lines from these patients must be generated from naïve T cells as in a primary response.

Generation of EBV-CTL lines from EBV negative patients using the standard protocols for EBV positive donors has generally been unsuccessful, therefore alternative methods have been developed. Two approaches have been shown to result in successful establishment of EBV cell lines. Stimulation of PBMC with autologous LCL followed by selection and restimulation of activated CD25 positive cells can be used to enrich for EBV-specific cells and to generate EBV-specific, CD4+, MHC class II restricted, cytotoxic cell lines (Savoldo*, et al* 2002). Alternatively, stimulation of PBMC by autologous LCL in the presence of the cytokines IL-12 and IL-7, rather than standard supplementation with IL-2, resulted in CD8+, class I restricted cytotoxic T cell lines (Comoli*, et al* 2006). As a result of these studies, adoptive immunotherapy with autologous EBV-CTLs can be available to a wider patient cohort including those EBV-negative individuals who are at highest risk.

One major drawback with the generation of autologous CTLs on a patient-by-patient basis is the considerable time required for *in vitro* expansion of these cells. Establishment of an LCL line occurs in approximately 4-6 weeks, after which an additional 4-5 weeks is required for EBV-CTL generation and testing. While this 10 week period may not be a consideration if CTLs are used prophylactically, it would

significantly limit the applicability of this approach for pre-emptive use or therapy of established PTLD. It has been proposed that this delay could be covered by reducing immunosuppression and administering Rituximab therapy to allow generation of a patient-specific therapeutic product (Savoldo*, et al* 2006). Alternatively, another approach developed to circumvent this delay is the use of pre-generated, allogeneic CTL lines from partially HLA matched third party donors.

1.4.2.3 Third party EBV-CTLs

In order to overcome the practical issues associated with generation of autologous cell lines on a patient-by-patient basis, Haque *et al* have established a well characterised bank of 100 EBV-CTL lines that are immediately available (Wilkie*, et al* 2004). Cells from this facility can be selected based on the closest HLA match between the patient and the cell product and shipped to the patient's treating hospital, obviating the need for specialist facilities and removing the lag time required for LCL and CTL generation. Despite the inevitable HLA mismatches, a phase II trial of this approach recently demonstrated complete responses in 12 of 33 treated patients, and partial responses in a further 9 patients all of whom were refractory to conventional treatment options. These responses were well maintained, with one complete and four partial responders relapsing but two partial responders converting to complete response. No patient experienced graft rejection or toxicity as a result of CTL treatment. It is important to note that immunosuppression was reduced in all patients in this trial and was stopped entirely in 26, although in some patients this was re-introduced prior to EBV-CTL infusion. The impact of these changes are difficult to assess but may have had the dual effect of both allowing the transferred EBV-CTLs to function unimpaired, whilst allowing the recipient's EBV-immunity to recover. However, along with recovery of EBV-specific immunity, it is possible that an allo-reactive response directed towards the therapeutic cells could be generated by the recipient, resulting in clearance of the infused CTLs. While persistence of the transferred cells was not systematically studied, the data available suggest that third party CTLs are undetectable within 4-6 weeks of the first infusion, possibly suggestive of

immunological clearance. This possibility is highlighted by the development of allo-antibodies directed towards the mismatched HLA antigens in one patient.

Overall, 52% of patients maintained a response six months post infusion, demonstrating a considerable benefit of EBV-CTL therapy in a patient group with advanced disease and otherwise poor prognosis (Haque*, et al* 1998, Haque*, et al* 2007). This approach is a promising alternative for patients who have failed reduction of immunosuppression or Rituximab therapy for whom autologous EBV-CTLs are not available, and is associated with significant response rates.

1.4.2.4 Effect of pharmacological immunosuppression on cell therapy

Although EBV-CTLs can be generated from immunosuppressed patients and expanded *ex vivo*, it can be concluded from the above studies that the remarkable success of immunotherapy for both CMV and EBV post stem cell transplantation has not been replicated following SOT. Two explanations are generally offered for the disappointing results achieved thus far. The first of these is that, unlike post-SCT where conditioning has "emptied" the immune compartment, leaving an immunological niche into which the cells can expand, EBV-CTLs administered post SOT enter a lymphocyte replete patient and must therefore compete to expand and establish a memory population. Another possibility is that the function of the adoptively transferred CTLs is compromised by ongoing immunosuppressive therapy in the SOT setting, whereas the majority of SCT patients received EBV-CTLs following cessation of immunosuppression. Indeed, Savoldo *et al* studied the effect of the two main immunosuppressants used to prevent graft rejection following SOT. The calcineurin inhibitors Tacrolimus (FK506) and Cyclosporin A (CsA) both inhibited proliferation, and profoundly reduced cytokine secretion from *ex vivo* expanded EBV-CTL lines (Savoldo*, et al* 2001). In addition, it has been shown that although CMV-specific CD8+ T cells are present at normal levels in patients receiving immunosuppressive therapy, these cells display impaired functionality as shown by reduced IFN-γ secretion (Engstrand*, et al* 2003).

As a result of the above evidence, we propose that the efficacy of adoptive immunotherapy for PTLD following SOT could be enhanced by the generation of EBV-CTL lines that are resistant to the suppressive effects of calcineurin inhibitors. This approach would remove the need for tapering immunosuppressive therapy and therefore the attendant risk of graft rejection, while allowing the infused cell product to function uninhibited. To this end, we have investigated the possibility of rendering EBV-CTLs resistant to calcineurin inhibitors by transduction with calcineurin mutants.

1.5 Generation of immunosuppression resistant CTLs for immunotherapy

1.5.1 Mechanism of action of CN inhibitors

Activation of T cells is critical in the induction of an immune response, inducing a complex interplay between helper and cytotoxic T cells, B cells and the innate immune system that is critical in generating efficient and appropriate responses. Therefore the interruption of cellular immunity by disrupting T cell activation is the focus of many immunosuppressive agents.

T cell activation is initiated by binding of cognate antigen, presented in the appropriate MHC molecule, to the T cell receptor (TCR). Adhesion molecules stabilise this immunological synapse, and costimulatory pathways must also be triggered to deliver co-ordinated signals to the nucleus of the T cell, resulting in effective activation. The most important of these being binding of CD28 on the T cell by CD80/86 on the target antigen presenting cell, although several other possible costimulatory pathways that can substitute for CD28. Following ligation of the TCR, several phosphatases are activated and initiate signalling through four main pathways. With help from the CD45 molecule, the Src family kinases Lck and Fyn dephosphorylate ZAP-70 and Syk, leading to activation of phospholipase C γ1 (PLCγ1). This results in hydrolysis of Phosphatidylinositol-5,4-bisphosphate (PIP_2) to diacyl glycerol (DAG) and inositol phosphate$_3$ (IP_3), simultaneously activating three pathways: the Ras pathway, resulting

in Erk translocation to the nucleus, protein kinase C θ (PKCθ) activation initiates the NFκB pathway (nuclear factor kappa light chain enhancer of activated B cells), and the Calmodulin/Calcineurin pathway activating the nuclear factor of activated T cells (NFAT). TCR ligation also triggers mitogen activated protein kinase kinase kinase (MEKK) signalling, which activates p38 and Jnk, allowing entry to the nucleus and the presence of activated Fos and Jun. Binding of CD28 leads to activation of PIP3, which in conjunction with PKCθ (activated by DAG), leads to activation and translocation of NFκB (Aringer 2002). As communication between pathways and cross-regulation is increasingly understood, it is becoming clear that the defined signalling "pathways" involved in T cell activation are in fact complex networks (Smith-Garvin*, et al* 2009). T cell activation remains under continued investigation, however a simplified illustration of the understanding of these pathways to date in shown in Figure 5.

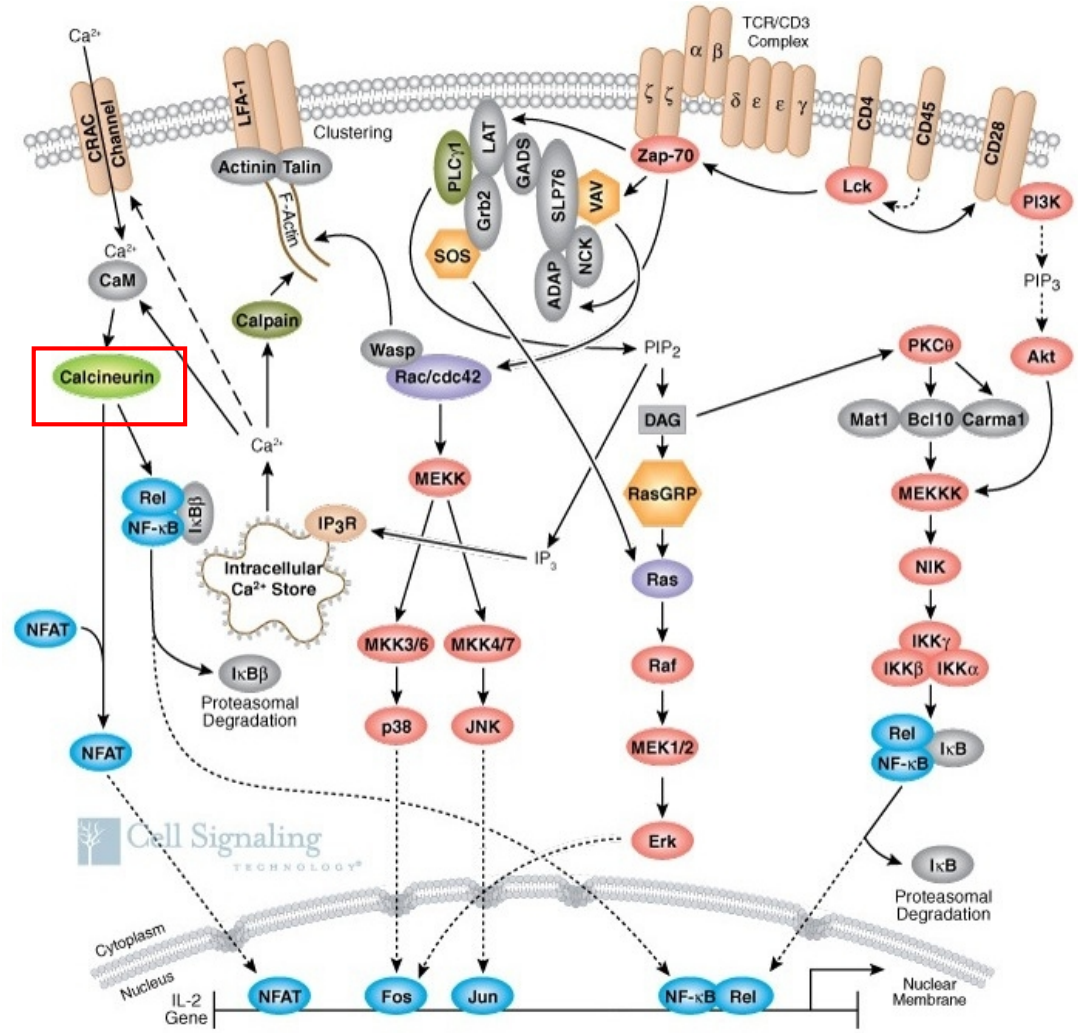


Figure 5: Illustration of T cell activation signal pathways. From Cell Signalling Technologies (http://www.cellsignal.com/reference/pathway/T_Cell_Receptor.html) Calcineurin is highlighted with red box.

As shown above, calcineurin (CN) is a key component of the calcium-regulated activation pathway in T cells. Calcineurin is a heterodimeric serine-threonine phosphatase, comprised of a catalytic subunit (CNa) and a regulatory subunit (CNb) whose primary substrate is the transcription factor Nuclear Factor of Activated T cells (NFAT). Dephosphorylation of NFAT exposes its nuclear localisation signal resulting in translocation to the nucleus, where it is involved in regulating the expression of multiple genes involved in cellular activation, the classical example of which is IL-2 but also including IL-3-5, IL-8, IL-13, IFN-γ, CD40L and others (Im and Rao 2004,

Kiani, *et al* 2000). In addition, CN is thought to have effects on other transcription factors including activation of JNK, activation of I_KB kinase resulting in NFκB activation, dephosphorylation of Elk-1 and indirect modulation of CREB. It can be concluded that CN is a pivotal molecule in activation of T cells, critical in activation of genes controlled by NFAT and also involved to a lesser extent in augmenting other activation pathways (Kiani, *et al* 2000).

The discovery of CN inhibitors in the 1980s revolutionised the field of transplantation medicine, allowing more effective suppression of the allo-specific immune response than was previously achievable, thus reducing rejection rates and improving survival post transplantation. Inhibition of CN activity results in profound suppression of T cell responses as normal activation signals are interrupted and T cells cannot respond to antigen binding. The CN inhibitors FK506 and CsA sterically prevent dephosphorylation of NFAT by binding to the composite face of CNa and CNb, inhibiting access to the active site by NFAT (Ho, *et al* 1996). Calcineurin inhibitors are currently the most commonly used class of immunosuppressive agents, which form the cornerstone of many SOT anti-rejection regimens. Although often used in conjunction with agents such as prednisolone, Mycophenolate Mofetil (MMF) and with additional treatment during rejection episodes, CN inhibitors are the key immunosuppressive agents used in transplantation medicine. In SOT patients who develop PTLD, other immunosuppressive agents such as MMF can often be safely discontinued but withdrawal of CN inhibitors frequently results in graft rejection (Webber, *et al* 2006). Therefore we have focussed on generating T cells that are resistant to suppression by FK506 and CsA.

FK506 and CsA are naturally occurring fungal metabolites which, despite their very similar effects and mode of action, are structurally distinct (see Figure 6). Both are bound by a cellular chaperone protein upon cell entry, the prolyl isomerases FK506 binding protein 12 (FKBP12) and Cyclophilin A (CyPA) respectively. Calcineurin was established as the intracellular target of FK506 and CsA, and a pivotal molecule in T cell activation in 1991 (Liu, *et al* 1991). The role of CN had been previously unknown and its importance in cell signalling became clear as a result of the effects of its inhibition. Neither the drugs or their chaperones are immunosuppressive

independently, but once bound the complex interacts with CN with extremely high specificity and sterically inhibits NFAT dephosphorylation, thus reducing this function of CN by several hundred to 1000 fold (see Figure 7) (Kiani, *et al* 2000).

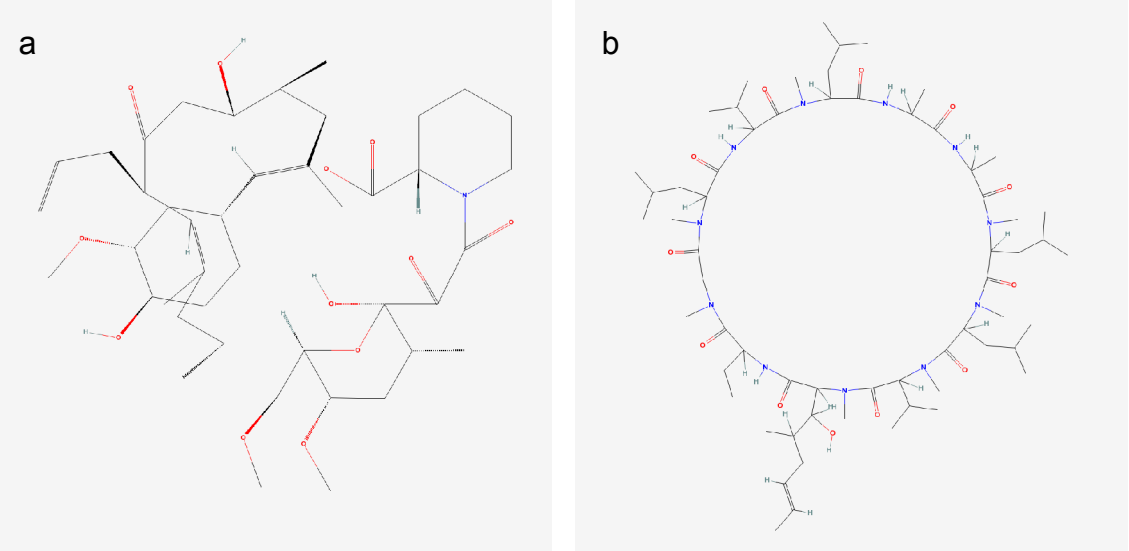


Figure 6: Structure of FK506 (a) and CsA (b). Similarities in mechanism of action and effects are not reflected structurally. Pubchem (http://pubchem.ncbi.nlm.nih.gov)

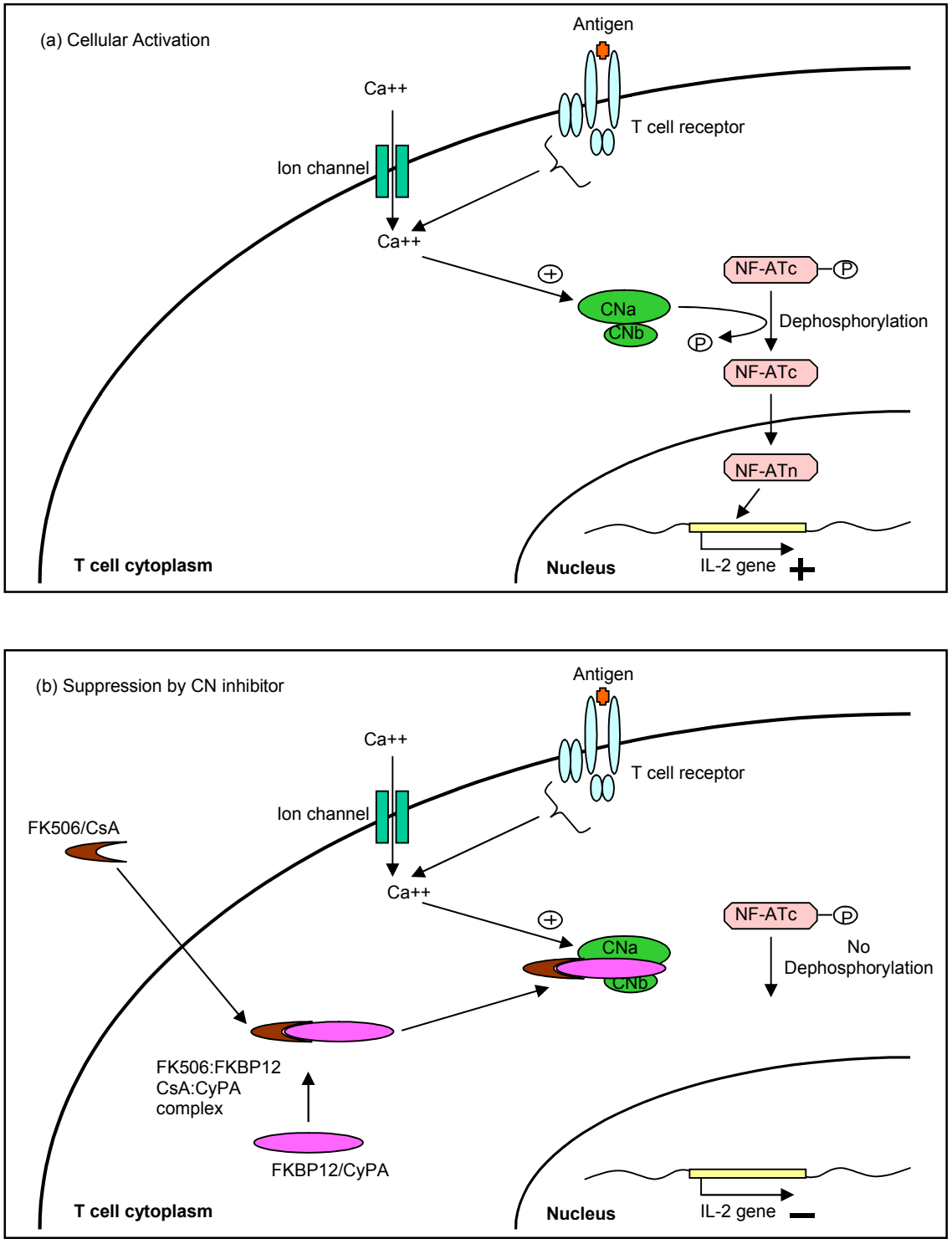


Figure 7: Schematic representation of CN inhibitor mechanism of action. (a) Normal cellular activation. (b) Binding of CN by FK506/CsA sterically inhibits NFAT dephosphorylation therefore preventing T cell activation.

1.5.2 Previously identified resistance to CN inhibitors

Calcineurin mutants with the ability to confer resistance to CN inhibitors have been reported previously in the literature. These studies focussed on the effect of CN mutants in yeast, with the aim of identifying the role of various residues of the CN molecule, rather than the potential utility of the CN mutation itself. Nevertheless, several CN mutants capable of conferring resistance to FK506, CsA or both have been identified.

In 1994, Milan *et al* identified two CNb mutants that conferred some resistance to both CN inhibitors in a yeast system, however these were also associated with reduced phosphatase activity. This study established the importance of CNb in both CN phosphatase activity and immunosuppressant binding, particularly highlighting the role of the C terminal latch region (Milan*, et al* 1994). Several studies followed to dissect the role of CNa and CNb regions in binding, activity and suppression of CN, with Kawamura identifying three CNa mutants that increased resistance to FK506 but not CsA, and Cardenas characterising three CsA and one FK506 resistant CNa mutant. (Cardenas*, et al* 1995, Kawamura and Su 1995). Although some of these studies were conducted in a yeast system, the results were reproduced showing resistance to FK506 or CsA in the human Jurkat cell line (Zhu*, et al* 1996). Finally, during investigation of a naturally occurring FK506 resistant strain of *C. neoformans*, a striking mutation was identified by Fox *et al* consisting of a six base pair duplication in the CNb gene. This results in a two amino acid insertion in the latch region whose importance had been previously established, and confers FK506 resistance while retaining CN activity (Fox*, et al* 2001).

This body of work provides insight into the mechanism of action of the both the CN molecule and CN inhibitors, and a basis for investigation of further FK506 or CsA resistant CN mutants. In addition to testing these published mutants for resistance to CN inhibitors in human cell lines, we have designed further mutations using the information gained from these studies regarding the importance of specific areas of the protein, together with predictions based on the crystal structure of the FKBP12/FK506/CN and CsA/CyPA/CN complexes (Griffith*, et al* 1995, Huai*, et al*

2002, Jin and Harrison 2002, Ke and Huai 2003, Kissinger, *et al* 1995). We have introduced these CN mutants into human T cells using retroviruses to confer resistance to CN inhibitors with the aim of generating cells that retain functionality in the presence of these immunosuppressive drugs.

1.5.3 Retroviral transduction of T cells

Gene transfer to human cells can be achieved using a variety of methods. However, as *ex vivo* expanded T lymphocytes proliferate rapidly, transduction with viral vectors remains the method of choice, ensuring integration into the genome and subsequent stable transgene expression. Several studies have been performed in which transduced T cells were administered to patients, the majority of which to date have utilised Moloney murine leukaemia virus (MLV) derived gamma retroviral vectors. Efficient gene transfer to human T cell lines can be achieved using MLV based vectors, with transduction efficiencies of up to 80-90% (Quintas-Cardama, *et al* 2007).

Due to serious adverse events observed in two clinical trials for X-SCID following transduction of haematopoietic stem cell (HSC) with retroviral vectors, research efforts have focussed on improving the safety of viral vectors. Development of leukaemia in these patients is attributed to activation of the proto-oncogene *LMO2* by nearby retroviral integration, followed by selective pressure favouring cells expressing the transgene and accumulation of further genetic abnormalities (Hacein-Bey-Abina, *et al* 2008, Hacein-Bey-Abina, *et al* 2003, Howe 2008). Integration of the viral genome has been shown to favour sites of active transcription, with integration "hotspots" near *LMO2* in stem cells, and read-through from the strong viral LTR promoter up-regulating expression of cellular genes. Thus, efforts to produce safer, self-inactivating (SIN) vectors with different integration profiles and weaker, endogenous promoters to reduce read-through have resulted in the SIN retro and lentiviral vectors (Schambach, *et al* 2007, Thornhill, *et al* 2008).

In contrast, retrovirally transduced mature T cells have been administered to over 100 patients with no adverse events recorded thus far, including 33 patients who received

neo^R marked EBV-CTLs post HSCT or Hodgkin's lymphoma. These patients have been followed up for over ten years, with persistence of infused cells demonstrated and no adverse events associated (Rooney*, et al* 1998, Roskrow*, et al* 1998). Several factors may explain this difference in safety profile of retrovirally transduced stem cells compared to T cells. First and most importantly T cells, in contrast to stem cells, are differentiated, thus limiting their potential for malignant transformation (Rossig and Brenner 2004). Furthermore, the pre-activation of HSCs that is necessary to allow retroviral transduction may force rapid cell division and hence predispose to the acquisition of genetic mutations, whereas transduction of EBV-CTLs can be performed using LCL stimulation which mimics physiological stimulation. In addition, transduced HSCs are introduced into the myelosuppressed patient, undergoing multiple rounds of proliferation and differentiation to reconstitute the immune system. This "empty" niche in which transduced cells have a selective advantage may be permissive environment for malignant transformation. Importantly, integration site analysis from *neo*^{R} marked EBV-CTLs demonstrates multiple different integration sites, mainly in non-coding regions, with no preference for proximity to oncogenes (Pule*, et al* 2004). Furthermore, retroviral transduction of T cells has been shown not to affect the Vβ usage of the T cell repertoire, the phenotype or the function of the treated cells (Coito*, et al* 2004, Ferrand*, et al* 2000, Recchia*, et al* 2006).

This evidence suggests that the use of retroviral vectors for transduction of mature T cells is a safe approach to gene transfer to facilitate adoptive immunotherapy. Our laboratory has considerable experience in the use of the MLV derived SFG retroviral vector (Riviere*, et al* 1995), which in addition has been utilised in several clinical trials. We have therefore used this vector to achieve gene transfer of CN mutants to EBV-CTL lines.

1.6 *In vivo* modelling of PTLD

Following the development of CN inhibitor resistant EBV-CTLs *in vitro*, the function of these cells was examined *in vivo* prior to the initiation of a clinical trial. The use of animal models in research is generally considered acceptable when the *in vitro* situation does not adequately reflect the wider biological environment, and where a suitable animal model exists. As the *in vivo* development and treatment of PTLD can not be modelled fully *in vitro*, we believe it is justified to use animal models to study this disease and evaluate new therapies.

1.6.1 Large animal models for PTLD

Previously, EBV infection was studied using primate models. Both new and old world primate systems are available and reflect both human EBV infection and PTLD development. Because of ubiquitous infection with simian herpesviruses, resulting in the production of cross-reactive antibodies, old world species are resistant to EBV infection. However, Rhesus monkeys are susceptible to rhesus gammaherpesvirus infection which produces an IM like illness, and these have been used to study primary infection (Johannessen 2002).

New world monkeys are not immune to EBV, and have been used to study both infection and PTLD. The common marmoset experiences an IM-like illness at primary infection with EBV without subsequent development of lymphoma, whereas the cotton top tamarind consistently develops PTLD-like large B cell lymphoma (Johannessen 2002).

Although these models provided useful early insights into EBV infection and immunity, the use of non-human primates poses considerable ethical and practical problems. In addition to the endangered status of new world monkeys, all these species are rare, costly, and require specialist facilities and expertise. The advent of appropriate mouse models enabled the study of EBV whilst circumventing these issues.

1.6.2 Mouse models of PTLD

Balb/C mice can be experimentally infected with murine herpesvirus 68 (MHV68), leading to an IM-like illness after approximately 3 weeks (Nash*, et al* 2001). In addition, MHV68 leads to spontaneous murine LPD development in 9% of animals, which can be increased to 60% by immunosuppression with CsA, mirroring the effect of EBV infection in humans (Sunil-Chandra*, et al* 1994).

The description in 1983 of the *prkdc* mutation producing the SCID CB17 mouse represented a considerable advance for the study of human tumorigenesis and treatment. SCID CB17 mice have a defect in DNA repair which prevents effective VDJ recombination, leading to a lack of functional T and B cells (Bosma*, et al* 1983). Since the first demonstration of human tumour cell engraftment into SCID mice by Reddy in 1987, this model has been widely used for studying cancer development and treatment *in vivo* (Reddy*, et al* 1987). Following depletion of NK cells, SCID mice readily accept engraftment of xenogenic PBMC or LCL tumours, either intraperitoneally (i.p.) or subcutaneously (s.c.), and represent an appropriate model for PTLD immunotherapy (Johannessen 2002, Wagar*, et al* 2000). Regression of established LCL tumours by autologous EBV-CTL lines was demonstrated by Lacerda *et al*, and this model has subsequently been utilised by several groups (Foster*, et al* 2008, Lacerda*, et al* 1996, Quintarelli*, et al* 2007).

Despite the presence of the *prkdc* mutation and reduced T/B cells, the defect in DNA repair in SCID CB17 mice is not complete, with up to one quarter of animals retaining some residual T or B cells. In addition, SCID CB17 animals have functional NK cells which must be depleted prior to human cell engraftment. In order to design improved models, the NOD-SCID and β_2-microglobulin knockout NOD-SCID strains were utilised. These mice allow more efficient engraftment of human cells, likely because of absent NK activity (Wagar*, et al* 2000). An additional SCID mouse strain developed in our laboratory is the triple knockout model (3KO), generated by a genetic back-cross of the RAG2$^{-/-}$/γ-chain$^{-/-}$ mouse (Goldman*, et al* 1998) with the naturally C5 deficient AJ mouse strain (M. Blundell, Personal Communication). The

resulting RAG2$^{-/-}$/γ-chain$^{-/-}$/C5$^{-/-}$ strain is devoid of T cells, B cells and NK cells and provides the ideal background for study of xenografted human tumour cell lines.

Triple knockout SCID mice were used to establish the LCL model of PTLD development at our institute. This model has been used to assess the efficacy of immunotherapy during administration of CN inhibitors with untransduced EBV-CTLs in comparison to those rendered resistant to CN inhibitors by transduction with CN mutants. Demonstration of improved CTL activity in the presence of CN inhibitors by transduced compared to untransduced CTLs without toxicity will provide the necessary scientific background required before proceeding to a clinical trial of CN transduced CTLs in the prophylaxis and treatment of PTLD in humans.

1.6.3 Measurement of tumours in vivo

Early studies of tumour development models *in vivo* assessed tumour progression by palpation of s.c. tumours, in conjunction with calliper measurement of accessible lesions. Measurement of i.p. tumours posed more of a challenge, with alterations in animal behaviour and death, followed by histological examination of tumour progression, often used as end points (Lacerda*, et al* 1996). These methods have been superseded in recent years by the growing use of bioluminescent imaging technology, which allows precise tracking and quantification of xenografted tumour cells. Serial measurement of bioluminescent signal represents a sensitive, non-invasive technique for monitoring tumour progression or regression (Foster*, et al* 2008).

Bioluminescence refers to the production of light by naturally occurring luciferase enzymes, released upon oxidation of luciferin substrates to non-reactive oxyluciferins (Berger*, et al* 2008b). Approximately 30 luciferase-luciferin systems have been identified from many animal species, the most widely used of which is from the common firefly *Photinus pyralis* (Chandran*, et al* 2009). Isolation of Firefly luciferase and purification of the D-luciferin substrate allowed application of this technology to scientific research. Luminescence has been utilised for many years as an *in vitro* reporter gene, easily detectable in luminometers due to its low background in

biological systems and concurrent low signal-to-noise ratio (Nguyen, *et al* 1988). The development of sensitive charged coupled device (CCD) cameras and subsequently the Xenogen IVIS imaging system has allowed application of bioluminescent imaging to *in vivo* systems (Contag, *et al* 1997, Contag, *et al* 1998).

Bioluminescent imaging has become widely used for *in vivo* monitoring, including application to the EBV-PTLD model by previous groups. These studies have shown serial tumour monitoring using Luciferase transduced LCL, demonstrating progression and regression upon treatment (Foster, *et al* 2008), as well as tracking of Luc transduced EBV-CTLs demonstrating homing to the tumour (Quintarelli, *et al* 2007). These studies establish that bioluminescent imaging does not interfere with the utility of the SCID mouse EBV-PTLD model.

Nevertheless, variation due to factors other than tumour growth is possible when using bioluminescent imaging, including luciferase transgene silencing, inadequate vascularisation of the region of interest, and methodological differences. Therefore, we have assessed these issues and established bioluminescent imaging as a technique to monitor both xenografted tumour and T cells in our triple knockout mouse model.

1.7 Alternative applications of CN inhibitor resistance

1.7.1 Immunotherapy post stem cell transplantation

The ability to render cells resistant to suppression by CN inhibitors could have benefits extending further than treatment of PTLD developing post SOT. The main alternative application of immunotherapy in the face of immunosuppression arises following SCT. T cell depletion associated with conditioning therapy together with post-transplant immunosuppression used to prevent GVHD render SCT patients at increased risk of infections for a window of several months post transplant. Hence during this period, viral infections, particularly with EBV, CMV and adenovirus are a major cause of morbidity and mortality. Adoptive immunotherapy represents a rational approach to prevention or treatment of these complications.

For EBV-associated PTLD, the majority of patients receiving EBV-CTLs as prophylaxis or treatment to date have had their immunosuppression tapered prior to infusion, to allow optimal function of the infused CTLs. However, some patients have previous or active GVHD at the time of development of PTLD, particularly when T cell specific agents such as ATG are used as therapy, thus precluding withdrawal of immunosuppression. In such circumstances, CN inhibitor resistant EBV-CTLs could confer protective immunity against EBV without the need to reduce immunosuppression and the attendant risk of increased GVHD.

Conversely, in some clinical trials of immunotherapy with CMV-specific CTLs following SCT, immunosuppressive therapy with CN inhibitors has been continued after infusion of the therapeutic product to control GVHD (Mackinnon*, et al* 2008). Whilst these studies have shown that the infused CTLs expand *in vivo* and may have some efficacy including viraemia and preventing CMV disease, it remains likely that ongoing immunosuppression curtails the function of the infused cell product to some extent. Furthermore, CTL cytotoxicity is thought to remain unaffected by CN inhibitors, therefore this may contribute to short-term disease control (Savoldo*, et al* 2001). Suppression with CN inhibitors *in vitro* prevents proliferation and cytokine secretion after 1-2 weeks culture; therefore cells may retain functionality in the short term following infusion. However, proliferation and establishment of memory responses in the longer term is likely to be compromised by ongoing immunosuppression, a problem which would be circumvented by rendering cells resistant to CN inhibitors prior to administration.

In addition to virus-related disease, T cell therapy may also be used to target leukaemia and other tumours, through conventional or chimaeric T cell receptor transfer (Morgan*, et al* 2006, Pule*, et al* 2008, Stauss*, et al* 2008). Immunotherapy directed towards tumour antigens to enhance the GVL effect following SCT, target residual disease and maintain long term remission is likely to be most effective if administered early post transplantation when the immune compartment is empty and the tumour burden is low. However, administration of GVHD prophylaxis is also most important in the immediate post transplant period. Therefore, the ability to render cells

resistant to CN inhibitors may allow continued protection from GVHD in parallel with efficient anti-tumour function by the infused CTLs.

The generation of CN inhibitor resistant CTLs may therefore represent a considerable advance in the treatment of EBV-PTLD after SOT, as well as for viral infections and a broader range of malignancies post SCT.

1.8 Project aims

- To design and generate a panel of CN mutants conferring resistance to FK506, CsA or both.
- To screen this panel of mutants in cell line assays to identify those retaining activity in the presence of CN inhibitors.
- To assess the selected mutants in retrovirally transduced normal donor EBV-CTL lines for the ability to confer resistance to CN inhibitors.
- To determine if transduction of EBV-CTLs with CN mutants alters their phenotype, antigen dependence or growth kinetics.
- To establish a xenogenic murine model of EBV-PTLD and optimise bioluminescent imaging.
- To test the efficacy of immunotherapy with transduced EBV-CTL lines in the face of immunosuppression *in vivo*.

Chapter two

Materials and Methods

2.1 Materials

2.1.1 Reagents

Unless otherwise stated, all tissue culture reagents were supplied by Gibco BRL (Invitrogen) and all general chemicals were supplied by Sigma.

General reagents and enzymes:

(methyl-^{3}H) Thymidine	TRK758 Perkin Elmer
1kb Plus DNA Ladder	10787-018 Invitrogen
^{51}Chromium labelled sodium chromate	NEZ030002MC Perkin Elmer
Agar	1.10283.0500 MERCK
Agarose	AM-9042 Invitrogen
Cyclosporin A (Sandimmune)	478941 Sandoz Pharmaceuticals
D-Luciferin	360221 Regis Technologies
ELISA substrate reagent pack	DY999 R&D Systems
Ficoll-Paque PLUS	17-1440-02 Amersham Biosciences
GeneJuice	70967-3 Novagen
Lipofectamine2000	11668-019 Invitrogen
Matrigel	354234 Beckton Dickinson
MeltiLex wax scintillation	1205-441 Perkin Elmer
Primocin	ant-pm-1 Invivogen
Recombinant human IL-2	Z00368 GenScript

Red Cell Lysis Buffer	00-4333 EBioscience
Restriction Endonucleases + buffers	New England Biosciences
Retronectin	T100B TaKara
Sucrose	BDH8029 BDH
Tacrolimus (Prograf)	119488 Astellas pharmaceuticals
Polybrene	AL-118 Sigma

Tissue culture media and supplements:

Medium	Supplements
Foetal Bovine Serum. (Sigma F7524)	
Complete DMEM (Invitrogen 61965-026)	10% FBS, 100U/ml penicillin, 100µg/ml streptomycin (Invitrogen 15140-122)
Complete RPMI (Invitrogen 61870-010)	10% FBS, 100U/ml penicillin, 100µg/ml streptomycin
Optimem (Invitrogen 31985-047)	No additions.
CTL media	45% hyclone RPMI (Hyclone SH30096.02), 45% Click's media (Irvine Scientific 9195), 20mM L-glutamine, 10% hyclone FBS (Hyclone SH30070.03), 100U/ml penicillin, 100µg/mlstreptomycin
Trypsin/EDTA (Invitrogen 25300-062)	
2x freezing medium	60% FBS, 20% DMSO (Sigma D2650), 20% complete RPMI.
IMDM (Sigma 13390)	10% FBS, 100U/ml penicillin, 100µg/ml streptomycin (Invitrogen 15140-122), 20mM L-glutamine.
AIM V (Invitrogen 12055-091)	No additions

2.1.2 Buffers and solutions

All buffers and solutions were prepared in double distilled water (ddH_20), sterile solutions were prepared in ddH_20, autoclaved for 15 minutes at 121^OC or sterile filtered through a 0.22μm filter. Composition of buffers and solutions is listed below.

Buffer	**Ingredients**
Orange G loading buffer (6x)	10mM Tris Ph 7.5, 50Mm EDTA, 10% Ficol 400, 0.4% Orange G (Sigma 861286))
Flow Cytometry staining buffer (FACS buffer)	PBS, 0.5% FBS
Flow cytometry fixing buffer (FACS fixing buffer)	FACS buffer + 0.5% Paraformaldehyde (Sigma 441244)
Luria-Bertani Broth (LB)	25g Luria Broth base powder (Invitrogen 12795-027) per litre dH_20. Ampicillin (Sigma A9393) was added at 10mg/ml where indicated (LB-Amp). Supplemented with 1.5% (w/v) Bacto-Agar for solid medium.
TAE (x50)	0.2M Tris, 1M acetic acid (BDH) 50mM EDTA, pH8.0
ELISPOT Acetate Buffer	4.6ml 0.1M acetic acid, 11ml 0.1M sodium acetate, 46.9ml ddH_20
APC Complex solution	PBS/ 0.1% Tween 20
ELISPOT Coating buffer	1.59g Na_2CO_3, 2.93g $NaHCO_3$, 200mg NaN_3, made up to 1L with sterile water pH 9.6, Sterile filtered 0.22μm
ELISA stop solution	2N H_2SO_4. 54ml concentrated H_2SO_4 in 1L dH_2O.

2.1.3 Kits

Purelink Miniprep	K2-10010 Invitrogen
Maxiprep	12165 Qiagen
QIAquick gel extraction kit	28704 Qiagen
QIAquick PCR purification kit	28104 Qiagen
DualGlo Luciferase assay	E2920 Promega
Duoset ELISA kits IL-2 and IFNγ	DY202 and DY285 R&D systems

2.1.4 Cell lines

293T	Human embryonic kidney cell line
B95-8	Marmoset cell line that releases high titres of transforming EBV (DSMZ cell bank ACC 100).
Jurkat	T cell line derived from non-hodgkin lymphoma patient. Kind gift from Dr Clio Rooney, Center for Cell and Gene Therapy, Houston, TX, USA.
HSB-2	LAK sensitive cell line derived from acute lymphoblastic leukaemia patient (DSMZ cell bank ACC 435).
LCL	Derived from normal donor B cells transformed with supernatant from B95-8 cells
CTL	Generated from normal donor PBMC stimulated with autologous LCL
FLYRD18	HT1080 derived cell line stably expressing moloney MLV GagPol and RD114 Env genes. (ECACC cell bank 95091902)

2.1.5 Antibodies

The following monoclonal antibodies were used in this study:

Flow cytometry:

Becton Dickinson: CD3-APC (555355), CD4-FitC (555346), CD19-PerCP (SJ25C1), CD25-APC (555434).

eBioscience: CD3-Pacific Blue (57-0037-73), CD4-Pacific Blue (57-0048-73), CD8-Pacific Blue (57-0086-73), CD16-PE (12-0168-73), CD56-PE (12-0569-71), CD62L-APC (17-0629-73), .

Serotec: CD45RO-PE (MCA461PET).

Western blotting:

Becton Dickinson: CNa (556350).

AbCam: CNb (ab49658), β-actin (ab49846).

ELISPOT:

MabTech: IFN-γ capture Ab (mAB 1-DIK), IFN-γ detection Ab (mAb 7-B6-1).

Multiscreen ELISPOT plates	MAHAS4510 Millipore
APC reagent kit	PK6100 Vector laboratories
AEC tablets	A6926 Sigma

Cytotoxicity assay:

Isoplate	1400-515 Perkin Elmer
OptiPhase ‘Supermix’ scintillation fluid	1200-439 Perkin Elmer
Plate sealer	0580 ABgene

2.1.6 Western Blotting

CelLytic Lysis buffer	C3228 Sigma
Protease inhibitor cocktail	P8340 Sigma
Sample buffer	NP0008 Invitrogen
Reducing agent	NP0009 Invitrogen
Anti-oxidant	NP0005 Invitrogen
SeeBlue Plus2 pre-stained standard	LC5925 Invitrogen
NuPAGE 4-12% novex Bis-Tris gels	NP0321 Invitrogen
MOPS Buffer	NP0001 Invitrogen
Transfer buffer	NP0006 Invitrogen
SuperSignal West Pico Chemiluminescent Substrate	34080 Pierce

2.1.7 PCR

Primers	IDT DNA
Phusion DNA Polymerase	F-530 Finnzyme, supplied by NEB
Phusion PCR buffer	F-519 Finnzyme, supplied by NEB
dNTPs	U1515 Promega
Quick Ligase	M2200 NEB
Quick Ligase Buffer	B2200 NEB

2.1.8 Bacteria

DH5α heat-shock competent bacteria	C2987H NEB

2.2 Methods

2.2.1 DNA manipulation

2.2.1.1 Small scale DNA preparation

Single colonies were picked from an agar plate and grown overnight in 5ml LB-Amp. The Invitrogen purelink miniprep kit was used to isolate plasmid DNA according to the manufacturer's instructions.

2.2.1.2 Large scale DNA preparation

500ml LB-Amp cultures were inoculated with a 5ml bacterial culture and grown overnight with shaking. Qiagen maxiprep kit was used as per the manufacturer's instructions to extract plasmid DNA from the resulting bacterial prep.

2.2.1.3 Measurement of DNA concentration

DNA concentration was calculated by measuring the absorbance of light with a wavelength of 260 nm (A_{260}) using a NanoDrop ND-1000 spectrophotometer with a 0.2 mm pathlength; at this wavelength 50 µg/ml of double-stranded DNA has an absorbance of 1.

2.2.1.4 Design and generation of mutants

Crystallographic data were used to identify critical residues involved in the interaction between Calcineurin and the FK506/FKBP12 or CsA/Cyclophilin A heterodimers. These were substituted with alternatives predicted to interrupt binding based on their charge or side chain characteristics.

Mutations were generated using overlap extension PCR. Two fragments of the CN gene adjacent to the target site were amplified in initial mutagenesis PCRs utilising one complimentary external primer and one internal primer containing the desired sequence change. 35 cycles of amplification were performed (40s 98^{o}, 30s 64^{o}, 90s 72^{o}) using Phusion polymerase. Products were purified using Qiaquick PCR clean-up columns and joined in a fusion PCR using the two external primers and amplification conditions above. External primers included AgeI and NotI restriction enzyme sites to facilitate subsequent subcloning. Following generation of mutants and subcloning into the SFG vector, CNa/b mutants were confirmed by capillary sequencing (Lark).

2.2.1.5 PCR purification

The product of the above PCR reactions was purified with the Qiagen QIAquick PCR clean-up column according to the manufacturer's instructions. A portion of the resultant product was examined by gel electrophoresis to confirm size and purity.

2.2.1.6 Restriction endonuclease digestion

A 20-fold over-digestion was performed to produce fragments for subsequent ligation or for confirmation of successful cloning. 0.5-5µg of plasmid DNA was digested in a final volume of 20-200µl. The recommended buffer was used, diluted to 1x with dd$H_2$0 and 0.1mg/ml BSA was added where required. The volume of enzyme varied according to concentration but never exceeded 10% (v/v) of the final reaction volume. CNa and CNb mutants generated as above were subcloned as AgeI-NotI fragments into the SFG_eGFP retroviral vector. Digestion was performed for 3 hours at 37^{o}C and verified by agarose gel electrophoresis. Double digestions were performed in parallel where buffers were compatible or sequentially following clean-up of DNA where buffers were incompatible using the Qiagen QIAquick clean-up kit.

2.2.1.7 Gel electrophoresis

The size of PCR products and digested plasmids was confirmed by gel electrophoresis. 1% agarose gels were prepared in 1x TAE buffer by boiling in a microwave. Once melted and cooled, 0.5µg/ml ethidium bromide was added to allow DNA visualisation. DNA samples were mixed with Orange G loading buffer at a ratio of 6:1 prior to addition to agarose gels. 1kb plus DNA ladder was included to allow determination of DNA fragment size. Gels were electrophoresed at 50-100V and up to 150mA in 1x TAE buffer until adequate separation of bands was observed. Fragments were visualised by exposure to UV light using the UVIdoc gel documentation system.

2.2.1.8 Gel extraction

Following identification of the correct bands by gel electrophoresis, the desired band was visualised using a UV transillumintor and excised from the gel using a clean scalpel blade. Gel extraction was then performed using the QIAquick gel extraction kit according to manufacturer's instructions.

2.2.1.9 Ligation

Following restriction enzyme digestion of vector and insert, ligations were performed using 100ng of vector DNA at a Vector:Insert molar ratio of 1:3 and 1:6. Ligations were performed at room temperature for 5-10 minutes using Quick Ligase (NEB), according to the manufacturer's instructions. 2µl of the ligated DNA was transformed into *E.coli* as described above for miniprep and confirmation of successful cloning.

2.2.1.10 Plasmids

The SFG plasmid was used for expression of wild type and mutant CN genes. SFG is based on the Murine Moloney Leukaemia Virus, with the transgene start codon at the

start site of the deleted viral *env* gene. This is followed by an internal ribosomal entry site (IRES), allowing expression of the downstream reporter gene eGFP. Fragments were cloned in using the unique AgeI site upstream of the transgene start site, and the NotI site immediately following the transgene termination codon. The SFG plasmid was also utilised to express FLuc_eBFP for transduction of LCLs used during subsequent *in vivo* experiments, where FLuc is followed by the foot and mouth disease 2A expression sequence instead of an IRES for expression of the reporter gene blue fluorescent protein.

2.2.1.11 Codon optimisation

An optimal sequence was designed using pMol software, based on optimisation of human codon usage, raising GC content to 70%, reduction of local hairpins and literal repeats to a minimum and avoidance of splice signals. The sequence was assembled with ligation-by-PCR using Phusion polymerase from overlapping oligonucleotides. The synthetic DNA was confirmed to be correct by capillary sequencing (Lark). This work was performed by Dr Martin Pule.

2.2.2 Bacterial manipulation

2.2.2.1 Growth and maintenance of E.coli

Escherichia coli (E.coli) were grown in liquid LB media with 10mg/ml ampicillin (LB-Amp) at 37^{o}C with agitation at 200rpm, or streaked out on solid LB-Amp agar plates supplemented with 1.5% Bacto Agar. For long term storage, bacterial cultures grown from a single colony were stored in 20% volume for volume (v/v) glycerol and stored at -80^{o}C.

2.2.2.2 Bacterial transformation

Competent DH5α *E.coli* were transformed by heat shock. 25µl competent bacteria were thawed on ice, 1-10ng DNA was added and incubated on ice for 30 minutes. Bacteria were transferred to a 42^{o} water bath for 35 seconds then placed on ice for 2 minutes. 250µl warm LB media was added and the cells were incubated at 37^{o}C with shaking for 1 hour. The resulting culture was streaked onto an LB-Amp agar plate and incubated at 37^{o} overnight.

2.2.3 Cell culture

2.2.3.1 Propagation of adherent cell lines

The adherent cell lines 293T and FLYRD18 were cultured in complete DMEM in 80 or 175cm^{2} tissue culture coated flasks at 37^{o}C with a 5% CO_2 atmosphere and passaged when 80-90% confluency was reached, approximately biweekly. Cells were washed with 1x PBS, incubated for 5 minutes at 37^{o}C with trypsin/EDTA, diluted 1:8-1:12 in fresh complete DMEM and transferred to new flasks.

2.2.3.2 Propagation of non-adherent cell lines

Non-adherent cell lines including Jurkat, LCL, B95-8 and HSB-2 were cultured in complete RPMI in upright 80cm^{2} tissue culture flasks at 37^{o}C with a 5% CO_2 atmosphere. Cells were maintained at a density of between approximately 0.2 and 1x10^{6}/ml, and passaged by 1:8-1:20 dilution with fresh medium when semi-confluent as determined by changing media colour.

2.2.3.3 Isolation of peripheral blood mononuclear cells (PBMC)

Whole blood obtained by venesection and anti-coagulated with preservative free heparin was diluted 1:1 with 1xPBS and 35ml layered onto 15ml Ficoll-Paque in 50ml centrifuge tubes. Tubes were centrifuged at 700g for 30 minutes at room temperature without brake, whereupon the white blood cell layer at the ficoll:plasma interface was removed. PBMC were washed x2 with PBS and resuspended in complete RPMI or CTL medium.

2.2.3.4 Generation of EBV supernatant from B95-8 cell line

The B95-8 cell line is derived from marmosets and continuously releases high titres of EBV. EBV preparations for transforming human PBMCs are prepared from this cell line in a CELLine flask, resulting in concentration of the produced virus. $2.5x10^7$ B95-8 cells are prepared in 15ml warm complete RPMI and inoculated into the cell compartment of the CELLine flask. One litre of warm RPMI containing 5% FCS is added to the nutrient compartment, separated from the cells by a nutrient permeable membrane. Cells are incubated for 7 days; whereupon they are harvested and old medium is replaced with 15ml fresh complete RPMI. The litre of RPMI with 5% FCS is replaced with 1L warm plain RPMI and the cells incubated for a further 7 days. Following this culture period, cells and media are collected from the cell compartment and centrifuged at 450g for 20 minutes; the supernatant containing the EBV virus is collected and centrifuged again under these conditions. The resulting supernatant is passed through a 0.45μm filter and frozen in 200μl aliquots at -80^oC for later use.

2.2.3.5 Generation of B-LCL lines

$5x10^6$ freshly isolated PBMC from healthy donors were centrifuged and resuspended in 200μl EBV supernatant produced from the B95-8 cell line. 1.8ml complete RPMI containing 1μg/ml Cyclosporin A (CsA) to inhibit EBV-specific T cell responses and

hence facilitate LCL growth was added and 200µl cells aliquoted into five wells of a flat-bottom 96 well tissue culture plate (96wp) ($5x10^5$/well). 1ml complete RPMI with CsA was added to the remaining 1ml cells and 200µl aliquotted to 10 further wells ($2.5x10^5$/well). The outer wells were filled with sterile distilled water and the cells were incubated at 37°C in 5% CO_2. The cells were incubated for one week, at which time half media changes were performed as necessary, determined by media colour, until cells were beginning to expand and clumps appeared. Subsequently, one well seeded with $5x10^5$ cells was combined with two seeded with $2.5x10^5$ cells in a 24wp and cells expanded for a further week, whereupon the LCL line was transferred to a vented 25cm^2 tissue culture flask.

2.2.3.6 Generation of EBV-CTL lines

EBV-CTLs were cultured in 45% Click's medium, 45% Hyclone RPMI and 10% Hyclone FBS supplemented with 20mM L-Glutamine, 100U/ml Penicillin and 100µg/ml Streptomycin (CTL medium). $2x10^6$ freshly isolated PBMCs were plated per well of a 24wp and stimulated with $5x10^4$ irradiated (40Gy) autologous LCL (ratio 40:1) in 2ml CTL medium. After 9-11 days, 10^6 viable cells per well were replated into 24wp and stimulated with $2.5x10^5$ autologous irradiated LCL (ratio 4:1). CTL were restimulated in this way weekly thereafter. 100U/ml IL-2 was added on day 13-14, replenished by resuspension in fresh IL-2 containing CTL medium at every weekly stimulation and by half-volume media changes containing 200U/ml IL-2 3-4 days after each stimulation. If CTLs became over-confluent, each well was divided into two and 1ml CTL media with IL-2 was added to each.

2.2.3.7 Cryopreservation of cell lines

Cells were cryopreserved for long term storage. Cells were centrifuged and resuspended in 0.5ml cold complete RPMI per $5-10x10^6$ cells. Cells and cryopreservation tubes were cooled on ice. An equal volume of cold 2x freezing

medium was added to the cells dropwise on ice with gentle agitation. 1ml was aliquoted to each cryopreservation tube and cells were transferred to -80^{o}C in a cooled isopropanol freezing container. This results in a controlled temperature decrease of -1^{o}C per minute. The following day, cells were transferred to the vapour phase of liquid nitrogen for long term storage. When required, cells were rapidly warmed to 37^{o}C and washed twice in warm complete RPMI.

2.2.4 Dual Luciferase Assay

293T cells were plated at 7x10^{4} per well in 24 well tissue culture plates in complete DMEM without antibiotics. The following day, cells were triple transfected with 400ng NFAT-RE_FLuc NFAT responsive Firefly Luciferase plasmid (Clontech), 8ng phRG-TK constitutively expressed Renilla luciferase control plasmid (Promega) and 600ng SFG-CN plasmid. The plasmids were combined and transfected into 293T cells using Lipofectamine2000 according to the manufacturer's instructions. Two days later, cultures were stimulated with 20ng/ml phorbol 12-myristate 13-acetate (PMA) and 1μg/ml ionomycin with or without 10ng/ml FK506 or 200ng/ml CsA. The next day, a Dual Luciferase Assay was performed in triplicate according to manufacturer's instructions and analysed using a FLUOstar Optima Luminometer (BMG Labtech). Transfected unstimulated and stimulated cells in the absence of CN inhibitors were used as negative and positive controls respectively. Firefly luciferase activity was normalised to Renilla luciferase activity to control for transfection efficiency. Results were expressed as percent activity of the stimulated sample for each construct without FK506/CsA (% resistance).

2.2.5 Production and titration of retrovirus

2.2.5.1 Production of transient retroviral supernatant

For Jurkat transductions, RD114 pseudotyped transient retroviral supernatants were generated by triple transfection of 4.69μg Peq-Pam plasmid (moloney GagPol), 3.125μg RDF plasmid (RD114 envelope), and 4.69μg SFG-CN plasmids into 293T cells using GeneJuice (Novagen). 1.5-1x10^6 293T were plated per 10cm tissue culture dish the day prior to transfection in 10ml complete IMDM. DNA was combined in 470μl plain DMEM and 30μl genejuice was added. Transfection mix was incubated for 15 minutes at room temperature, before dropwise addition to cells. Supernatant was harvested after 48 and 72 hours, combined and snap frozen in a bath of dry ice and ethanol for storage at -80^{o}C.

2.2.5.2 Production of stable retrovirus producer lines

High titre producer lines were generated by multiple transduction of FLY-RD18 cells with GALV pseudotyped SFG retrovirus containing CN mutants, produced as above. FLY-RD18 cells were transduced daily for 2 weeks, bulk FACS sorted for high expression of eGFP and single cell cloned by limiting dilution. This work was performed by Dr Martin Pule. Supernatant from the expanded clones was titred on 293T cells to identify the highest titre producers. These stable producer lines were used to generate high titre retroviral supernatants for transduction of EBV-CTLs.

2.2.5.3 Titration of retroviral supernatant

Retroviral supernatants were titrated on 293T cells. 2x10^4 cells were plated per well of a 24wp the day prior to titration. Six doubling dilutions of viral supernatant from 100 to 3μl were prepared in 200μl media and added to cells. Polybrene was included to a final concentration of 5μg/ml. Cells were incubated for 3 days when the cells were

harvested and analysed by flow cytometry for expression of the GFP reporter gene. Viral titre (infectious particles per ml) was calculated by multiplication of the number of transduced cells by the dilution.

2.2.6 Retroviral transduction

2.2.6.1 Retroviral transduction of cell lines and CTLs

Non-tissue culture treated 24wp were coated for 2 hours at 37^{o}C with 7μg/ml Retronectin in PBS and washed once with PBS. In some experiments, RN was pre-loaded with 0.2-0.5ml retroviral supernatant for 30 minutes at room temperature prior to addition of cells. 0.5x10^{6} Jurkat cells or CTLs were added per well in 0.5ml media, along with 2ml retroviral supernatant. Plates were spun for 40 minutes at 1000g and incubated at 37^{o}. 100U/ml IL-2 was added to EBV-CTLs, which were transduced 3 days after their second stimulation.

2.2.6.2 Assessment of transduction efficiency

Transduction efficiency was assessed by flow cytometry. Jurkat cells or EBV-CTLs (three or seven days after transduction respectively) were harvested with cell dissociation buffer, washed with PBS and 2x10^{5} cells resuspended in 300μl FACS buffer. Cells were analysed for expression of the GFP reporter gene using the FitC channel of a CyAn flow cytometer (Dako). Untransduced Jurkat cells or CTLs were used as negative controls and SFG_eGFP alone transduced cells as positive controls.

2.2.6.3 FACS sorting of transduced cells

Transduced Jurkat cells were sorted to ensure expression of the same mean fluorescence index (MFI) of GFP. 10x10^{6} cells were prepared in 1ml PBS with 1% FBS. FACS sorting was performed by flow cytometry operator Ajad Eddaoudi.

Expression of GFP was standardised, cells expressing GFP within a window containing the highest MFI represented in all cultures were selected. After sorting, Jurkats were cultured in complete RPMI containing 20% FCS and 1% Primocin for one week to encourage growth and prevent contamination.

2.2.6.4 Western blot analysis of CN expression

$5x10^6$ Jurkat cells or $2x10^6$ EBV-CTLs were harvested and washed twice in PBS. Cell pellets were resuspended in 125µl CelLytic lysis buffer containing 10% v/v Protease Inhibitor Cocktail and agitated at room temperature for 15 minutes. Samples were spun at 100g for one minute in a microcentrifuge and clear supernatant was removed to a chilled 1.5ml microcentrifuge tube. Samples were frozen at -20°C or run immediately. 10µl sample lysate was combined with 4µl sample buffer and 1.5µl reducing agent, boiled for 15 minutes at 70°C and centrifuged at 100g for 1 minute. Samples were kept on ice until loading. Sample were loaded onto pre-cast 4-12% Bis-tris polyacrylamide Novex gels and run in XCell *Sure-Lock* Mini-Cell (Invitrogen) with 1x NuPage MOPS SDS running buffer at 200V for 1-1½ hours with 500µl antioxidant (Invitrogen) in the central chamber. SeeBlue Plus2 pre-stained protein ladder was included in the outermost wells. The separated proteins were transferred onto Polyvinylidene Difluoride (PVDF) membrane using the XCell II blot module and 1x transfer buffer at 25V for 1 hour. Prior to protein transfer, the PVDF membrane was immersed in methanol for 30 seconds, and soaked in 1x transfer buffer for 5 minutes.

After transfer, the PVDF membrane was blocked with 5% BSA in PBS with agitation for at least one hour at room temperature, or overnight at 4°C to prevent non-specific antibody staining. The membrane was washed for 10 minutes with agitation three times at room temperature and subsequently incubated with primary antibody in 5ml 2.5% BSA in PBS for 2 hours with agitation at room temperature, or overnight at 4°C. Primary antibodies were used at the following dilutions: CNa=1/1000, CNb=1/2000, β-actin=1/4000. Membrane was washed again and incubated with secondary rat anti-mouse antibody, 1/2500 dilution in 5ml 1% BSA in PBS for 45minutes at room

temperature with agitation. 1ml ECL Western Blotting substrate was added to the membrane and incubated for 2 minutes at room temperature. Protein bands were visualised and quantitated using UVIchemi (UVItech) chemiluminescence documentation system and UVIsoft (UVItech) software. Membranes were cut into sections according to protein size, and the appropriate section was stained for β-actin was used to ensure comparable protein loading between samples.

2.2.7 Assessment of IL-2 secretion from Jurkat cells

Jurkat cells cultured in RPMI were split 1/10 the day prior to stimulation. The next day, $5x10^5$ Jurkat cells were plated per well of a 48wp and stimulated with 20ng/ml PMA and 1μg/ml Ionomycin. FK506/CsA was added at varying concentrations (0.5-40ng/ml and 50-800ng/ml respectively). Cells were incubated for 24h, when samples of supernatant were harvested and frozen at -20^{o}C for ELISA. To measure IL-2 secretion, the IL-2 DuoSet ELISA was used according to manufacturer's instructions and read using a FLUOstar Optima. To enable quantitation of IL-2 concentration, a standard curve was included using concentrations of IL-2 between 15 and 1000pg/ml. In most experiments the assay was linear between 60 and 1000 pg/ml.

2.2.8 Testing of EBV-CTL lines

2.2.8.1 ELISPOT assessment of IFN-γ secretion

To assess IFN-γ release from EBV-CTLs in response to stimulation with autologous LCL, a multiscreen ELISPOT plate (Millipore, MAHAS4510) was coated overnight with 100μl per well 10μg/ml capture antibody in coating buffer. Wells were washed x3 with 150μl PBS for five minutes and blocked with 150μl complete RPMI for at least one hour. CTLs (resuspended at $5x10^5$/ml in AIM V) or PBMC (resuspended at $2x10^6$/ml) from the same donor were used as responders. Four doubling dilutions of responder cells were prepared and 100μl CTLs ($5x10^4$, $2.5x10^4$, $1.25x10^4$, $6.25x10^3$)

or PBMC ($2x10^5$, $1x10^5$, $5x10^4$, $2.5x10^4$) were added per well in triplicate along with 10^5 autologous or allogeneic irradiated (40Gy) LCL. The plate was incubated undisturbed at 37°C/5% CO_2 for 18-20 hours. Triplicate controls consisting of responders or stimulators alone were included. After overnight incubation, cells were removed and the plate washed six times with PBS/0.05% Tween. Biotin conjugated detection antibody was prepared at 1μg/ml in PBS/0.5% Tween, 100μl was added per well and incubated at 37°C for two hours. The plate was washed and Avidin-peroxidase complex was added for one hour at room temperature. Spots were developed with 3-amino-9-ethylcarbazole substrate mix (AEC, Sigma). The number of spots was counted using an ELISPOT plate reader (Bioreader 3000). The mean number of spots in triplicate wells was calculated, with subtraction of the mean from control wells, and results expressed as specific spot forming cells per 10^5 effectors.

2.2.8.2 ELISA assessment of IFN-γ secretion

24 hours after the 6th stimulation, samples of supernatant were taken from EBV-CTL cultures and frozen at -20°C. IFN-γ concentration was measured using the IFN-γ DuoSet ELISA kit according to the manufacturer's instructions. A standard curve was included with samples from 15pg/ml to 1000pg/ml, the linear range of the assay was 60-1000pg/ml in most experiments.

2.2.8.3 ^{3}H-thymidine uptake proliferation assay

On the day of stimulation 6, 10^5 CTLs were plated in triplicate in U bottomed 96wp, with or without stimulation by $2.5x10^4$ autologous irradiated LCL (responder:stimulator ratio 4:1), in the presence or absence of 10ng/ml FK506 or 200ng/ml CsA. After 4 days, wells were pulsed with 1μl (37Bq) ^{3}H-thymidine and incubated for a further 19-20 hours, when cells were harvested to a filter mat using a Tomtec MachIIIM Harvester 96. MeltiLex wax scintillation was applied, and thymidine incorporation measured using a Wallac 1450 MicroBeta trilux beta-counter

(Perkin Elmer). Specific proliferation was calculated by subtracting mean counts per minute (cpm) of CTL and LCL alone control wells from those containing CTLs stimulated with LCL.

2.2.8.4 ^{51}Cr-release cytotoxicity assay

Cytotoxicity of EBV-CTL lines was determined using a chromium release assay. $2x10^6$ HSB-2 cells or autologous/allogeneic LCL targets were incubated at 37^o with 1mCi ^{51}Cr labelled sodium chromate for 1 hour, washed three times with RPMI, and counted. $5x10^3$ target cells were co-cultured with EBV-CTLs in triplicate in V-bottomed 96wp at effector:target ratios of 30:1, 15:1 and 7.5:1 with or without 10ng/ml FK506 or 200ng/ml CsA. Target cells alone in complete media or lysed with 1% TritonX-100 were used to determine spontaneous and maximum release respectively. Plates were centrifuged at 180g for three minutes before and after 4-6 hours incubation at 37^{o}C. 50µl supernatant was harvested, mixed with 150µl scintillation fluid in isoplates (Perkin Elmer), which were sealed and γ-irradiation (counts per minute) read using a Wallac 1450 MicroBeta trilux. Percent specific lysis was calculated as follows: (specific release – spontaneous release)/(maximum release – spontaneous release).

2.2.8.5 Flow cytometric analysis of EBV-CTL phenotype

Four days after the 5th stimulation with autologous LCL, $2x10^5$ CTLs per tube were washed in PBS and incubated in 100µl FACS buffer with monoclonal antibodies for 20 minutes at 4^{o}C in the dark. Cells were washed twice with 3ml cold FACS buffer and resuspended in 400µl FACS buffer for analysis. CTL lines were assessed for expression of surface markers using the following monoclonal antibodies: CD3, CD4, CD19, CD25 (Becton Dickinson), CD45RO (Serotec), CD8, CD16, CD56, CD62L (eBioscience). Appropriately matched isotype controls were included. Cells were analysed on an LSRII flow cytometer (Becton Dickinson).

2.2.8.6 Antigen dependence

To confirm that transduced EBV-CTLs remained dependent on stimulation with specific antigen, following 4 stimulations with autologous LCLs, CTLs were cultured without further stimulation with LCL and cell growth was monitored thereafter. In some cultures, addition of 100U/ml IL-2 twice weekly was continued. CTLs were counted weekly and re-plated at 10^6 viable cells per well of a 24wp.

2.2.9 In vivo investigations

2.2.9.1 Mouse strains

RAG2$^{-/-}$/γ-chain$^{-/-}$/C5$^{-/-}$ triple knockout mice as described in section 1.6.2 were obtained from a breeding colony maintained in our institute and housed in individually ventilated cages. Autoclaved food, water and bedding were utilised for these animals and mice were handled under aseptic conditions at all times. Ethical approval for murine work was obtained as per project licence number PPL70_5249.

2.2.9.2 Subcutaneous injection of LCL

10^7 LCL transduced with the FLuc_eBFP firefly luciferase retroviral vector per animal were washed three times with PBS and resuspended in 50µl cold plain RPMI. This was mixed with 50µl cold Matrigel and loaded into a 0.5ml 27 gauge (G) tuberculin syringe. Animals were restrained by scruffing and cells were injected subcutaneously on the right flank.

2.2.9.3 Monitoring of LCL tumours

The size of LCL tumours was assessed with callipers bi-weekly, in addition to weekly monitoring with bioluminescent imaging. For imaging, anaesthesia was induced by inhalation of isofluorane with oxygen. 2mg D-Luciferin was injected intraperitoneally in 200µl volume and animals were placed in the imaging chamber. Anaesthesia was maintained by delivery of isofluorane and oxygen to individual animals within the

imaging chamber. A region of interest (ROI) was delineated around the tumour site and images were captured at two minute intervals until the signal from each ROI plateaued. Animals recovered individually in warmed chambers. Total light counts within each ROI is measured and used along with exposure time to calculate the counts per second emitted by each tumour area.

2.2.9.4 Administration of EBV-CTLs

10^7 cryopreserved EBV-CTLs from the same donor as the LCL were thawed the day prior to injection, washed and rested overnight in 5ml CTL medium with 20U/ml IL-2 at 37°C/5% CO_2. The following day, CTLs were spun and resuspended in 100µl plain RPMI and transferred to a 0.5ml syringe. Animals were warmed at 37°C for 10 minutes and CTLs were injected into the tail vein using a 27G needle. Pressure was applied to the injection site following administration to reduce bleeding.

2.2.9.5 Administration of IL-2

2500U recombinant human IL-2 was administered intraperitoneally in 200µl PBS three times per week using a 0.5ml, 27G tuberculin syringe. This was performed concurrently with D-luciferin administration where appropriate to minimise the number of injections given.

2.2.10 Statistical analysis

Proliferation, IFN-γ secretion and phenotype of transduced and UT CTLs in the absence of CN inhibitors were compared using a one-way analysis of variance (ANOVA). Proliferation and IFN-γ secretion of transduced CTLs in the presence of CN inhibitors was compared to that of UT CTLs in the absence of CN inhibitors using a one-way ANOVA with the Dunnett's post-hoc test. Enrichment of GFP +ve CTLs was assessed using the paired t-test. Cytotoxicity was compared between transduced and UT CTLs in the presence or absence of CN inhibitors using a two-way ANOVA. Analysis was performed using SPSS statistical package (v16).

Chapter three

Design of CN mutants, evaluation in cell lines

3.0 Aims

- To design and generate a panel of CNa and CNb mutants to confer resistance to FK506 or CsA.
- To assess resistance from these mutants in cell line screening assays.
- To determine mutants conferring optimal resistance for further study in primary T cells.

3.1 Introduction

Calcineurin inhibitors specifically disrupt CN activity in T cells, preventing transduction of the activation signal from the engaged T cell receptor to the nucleus thereby preventing T cell activation. Suppression of T cells by the CN inhibitors FK506 and CsA is mediated through binding to a common surface on the CN heterodimer, the location of which results in blocking of the active site. The composite face of CNa and CNb provides the docking site for both the FK506:FKBP12 and CsA:CyPA complexes as they sterically inhibit access to the active site for NFAT. Therefore, this region provides an attractive target for mutagenesis, with the potential to disrupt the binding of either or both of these drugs. While other possibilities have been suggested (Matsuda*, et al* 2000), binding of these drugs to CN is widely thought to be their predominant mechanism of action, therefore prevention of this binding should confer complete resistance to suppression by FK506 or CsA (Ho*, et al* 1996).

As discussed in section 1.5.2, previous studies have identified naturally occurring mutations in CN that can confer a degree of resistance to either FK506 or CsA in yeast. The information gleaned from these investigations provides a useful base for design of additional and combination mutants. Following the generation of a panel of CN mutants, a screening assay was utilised to identify those to pursue in primary cell

work. We have established and optimised this assay and utilised it to select CN mutants that may confer resistance to therapeutic levels of FK506 or CsA in the Jurkat T cell line.

3.2 Design and generation of CN mutant panel

3.2.1 Design of CN mutants

In order to generate CN mutants able to resist binding by CN inhibitors, we combined previously published information regarding resistance in yeast with detailed structural data. These structural data provided information regarding both the conformation of CN and those residues that are likely to be critical in the interaction between CN and the IS:immunophilin complexes (Griffith*, et al* 1995, Ke and Huai 2003). We were able to identify several regions likely to be involved in this interaction and focussed on these when designing mutants. Published mutations shown to confer resistance to either FK506 or CsA were generated, along with combination mutants and also variations of these mutants with substitution for a different amino acid (AA). In addition, we generated several novel mutants based on the crystal structure of CNa/b in complex with either FK506/FKBP12 or CsA/CyPA.

Each targeted residue was substituted with either a conservative or a radical mutation. Conservative mutations replaced the original AA with another possessing similar characteristics, for example Serine with Asparagine at CNa 353 (mutant CNa6), or the reverse N122S in CNb7, whereas radical mutations replaced the original AA with one from a different group, or with a contrasting side chain eg Valine at position CNa 314 is a hydrophobic AA with a moderately sized side chain, this is substituted with either an amphipathic (Lysine), a hydrophilic (Arginine) or another hydrophobic residue (Phenylalanine) to produce CNa18-20. These alterations insert a residue containing a larger side group than the original, in addition some mutations alter the charge characteristics which at this critical residue could be expected to affect CN inhibitor binding. Data from Ke and Huai suggest that this residue is likely to be of importance in the interaction with both W121 of CyPA and K35 of FKBP12, therefore alterations

here have the potential to disrupt interactions with both FK506 and/or CsA (Ke and Huai 2003).

In addition to various single AA substitutions, and based on the identification of naturally occurring yeast mutants that are resistant to FK506, we investigated the generation of CNb mutants containing a two AA insertion following residue K125. As shown by Fox *et al*, insertion of VQ at this location confers 50% resistance to FK506, therefore we generated mutants containing either these residues or insertion of IE or LA at this site. This insertion was also combined with substitution mutants to create a second generation of constructs. When examining the crystal structure of CNb in this region, it seems likely that insertion of amino acids following K125 may disrupt secondary structure of the C terminal loop of CNb latch region, which is important in FK506 binding. The insertion of these two residues appears to distort this loop which links two α-helices, such that it protrudes outwards into the region where FK506 binds. We therefore incorporated insertions at this site into several of our panel of mutants. In contrast, CsA does not contact the latch region in such close proximity therefore CsA resistance is not anticipated from insertions at this location (Fox*, et al* 2001).

Generation of the panel of mutants was an ongoing process, whereby knowledge gained from testing earlier batches guided engineering of subsequently designed mutants. Several combination mutants were generated incorporating changes that conferred some effect in earlier builds, therefore resistant mutants evolved throughout the design phase. In total 22 CNa mutants and 32 CNb mutants were designed covering various regions of the components of the CN heterodimer. The distribution of these mutants is shown in Figure 8. Location of CNa and CNb mutants are shown separately in panels (a) and (b), followed by the assembled heterodimer in (c). Panels (d) and (e) show binding of CN by FKBP12:FK506 and CsA:CyPA respectively. It can be seen that the mutants generated are located on the composite binding face of CN, making it likely that docking of the immunosuppressive complexes may be disrupted. Details of the AA changes made for all mutants are shown in Table 4.

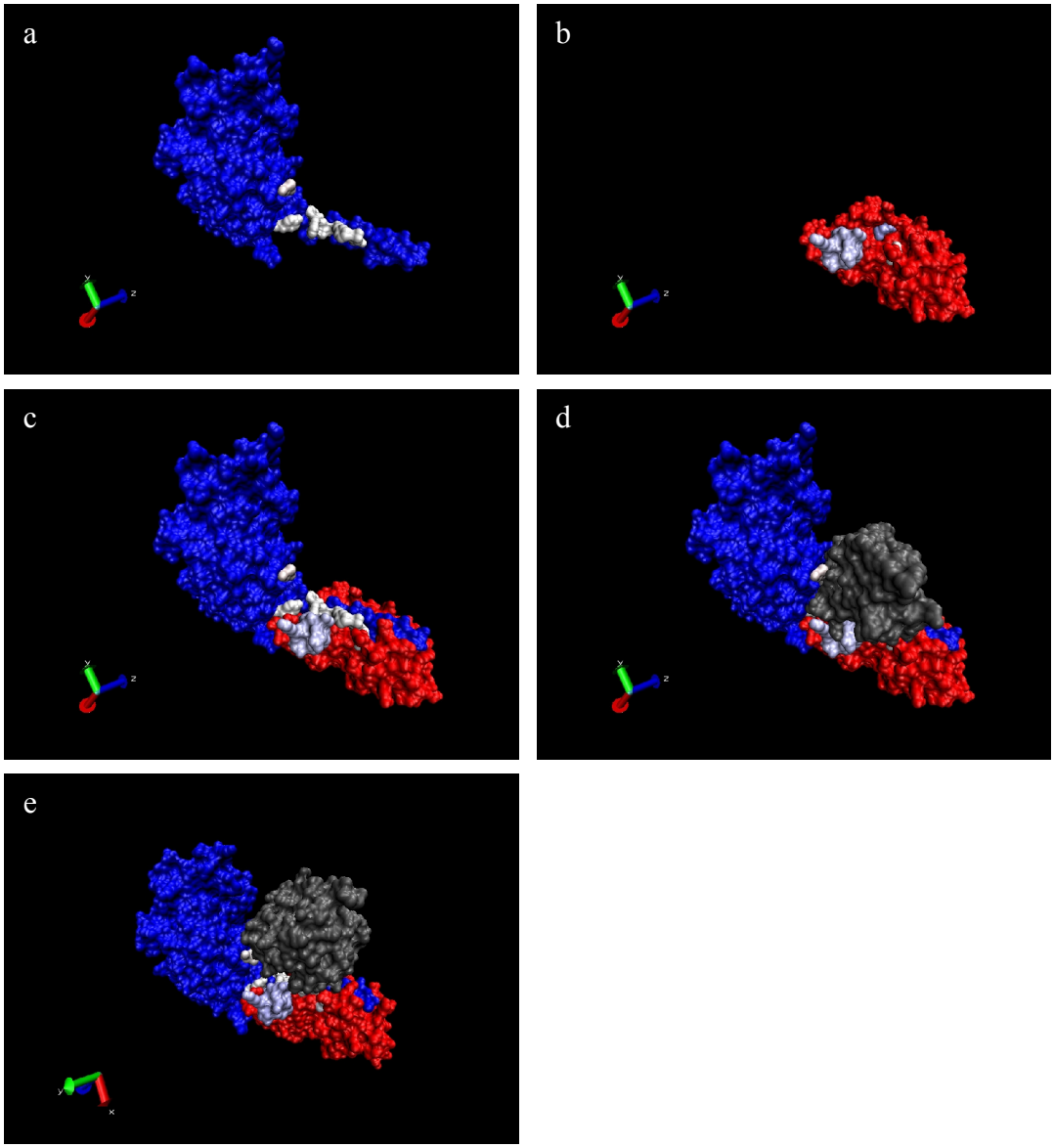


Figure 8: Location of CN mutations. a) CNa and b) CNb chains projected separately with location of mutations highlighted. c) CNa/CNb heterodimer showing binding face for FKBP12/FK506 or CyPA/CsA with location of mutations highlighted. d) CN heterodimer bound by FKBP12. e) CN heterodimer bound by CyPA. CNa shown in blue, CNb in red and FKBP12/CyPA in grey. Mutated residues in CNa are highlighted in white, in CNb are highlighted in silver.

Table 4: Mutations in CNa and CNb. Amino acid changes were introduced to generate a panel of 54 CN mutants designed to confer resistance to CN inhibitors.

Construct	Mutations
CNa1	L354A; K360A
CNa2	L354A; K360F
CNa3	T351E; K360F
CNa4	W352A
CNa5	S353H
CNa6	S353N
CNa7	F356A
CNa8	W352A; S353H
CNa9	W352C
CNa10	W352E
CNa11	K360F
CNa12	T351E; L354A
CNa13	W352C; K360F
CNa14	W352C; L354A; K360F
CNa15	M347W
CNa16	M347R
CNa17	M347E
CNa18	V314K
CNa19	V314R
CNa20	Y341F
CNa21	V314K; Y341F
CNa22	V314R; Y341F

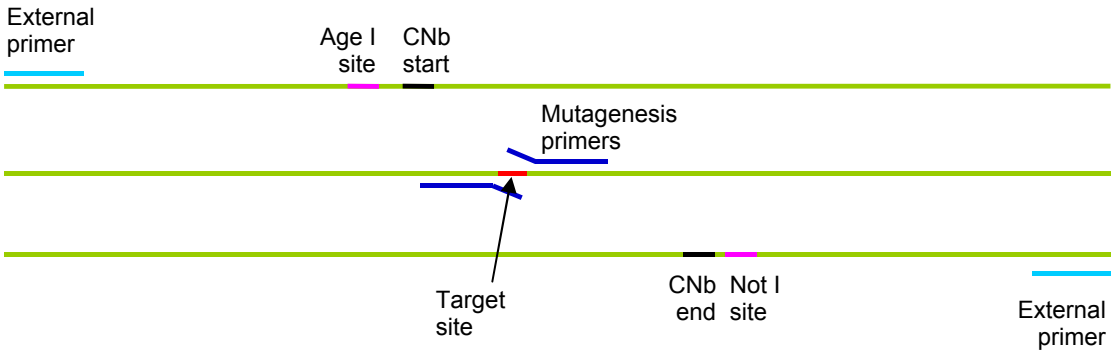
Construct	Mutations
CNb1	K125A
CNb2	K125E
CNb3	K125W
CNb4	N122A
CNb5	N122H
CNb6	N122F
CNb7	N123S
CNb8	N123H
CNb9	N123R
CNb10	N123F
CNb11	N123K
CNb12	N123W
CNb13	Q51S
CNb14	K165Q
CNb15	M119A
CNb16	M119W
CNb17	L116R
CNb18	L116Y
CNb19	V120L
CNb20	V120F
CNb21	K125-VQ-Ins
CNb22	K125-IE-Ins
CNb23	K125-LA-Ins
CNb24	V120S
CNb25	L124T
CNb26	V120S; L124T
CNb27	V120D
CNb28	V120D; L124T
CNb29	N123W; K125-LA-Ins
CNb30	L124T; K125-LA-Ins
CNb31	V120D; K125-LA-Ins
CNb32	M119-F-Ins; G121-LF-Ins

3.2.2 Generation of CN mutants

Site directed mutagenesis was performed using overlap extension PCR, with the desired sequence change contained within overlapping primers at the target site. Two initial PCRs were performed using complementary external primers, and internal primers containing these changes. The products of these two reactions were combined in a fusion PCR to join and amplify the desired final sequence. Template DNA for generation of CN mutants was wild type CNa (obtained from Dr. Pule) or CNb (cDNA derived from Jurkat cells and confirmed by sequencing).

Primers for mutagenesis reactions were designed to contain the specific change, whilst considering several other parameters: length, GC content and melting temperature (Tm) were standardised, with sufficient overlap to prime the subsequent fusion PCR. pMol software written by Dr. Pule was utilised to design many of these primers. An example of this strategy is shown in Figure 9, using wtCNb as template DNA to produce mutant CNb1. The products of these fusion reactions were AgeI-NotI subcloned into the SFG-eGFP retroviral vector, followed by sequencing to confirm that the desired sequence change was present and that no other alterations had been introduced during the amplifications (see Figure 10 for an example of sequencing).

(a)



(b)

aggtattgaagatgatggtggggaacaatctggccgataca

aggtattgaagatgatggtggggaacaatctgaaagatacacagttacagcaaattgtagacaaaaccataataaatg

ctggccgatacacagttacagcaaattgtagacaaaaccataataaatg

Figure 9: Illustration of mutagenesis strategy. (a) Illustration of generation of CN mutants. Initial PCRs utilise one external primer (pale blue) and one internal primer containing the desired sequence change (dark blue). Subsequent fusion PCR joins product of initial PCRs to generate the final product. Restriction enzyme sites for subcloning products into the SFG retroviral vector are indicated in pink. (b) Detail of target site and overlapping primer sequences to generate desired change, aaa->gcc for CNb1. Mutagenesis primers shown in blue, template DNA in black and target codon in red.

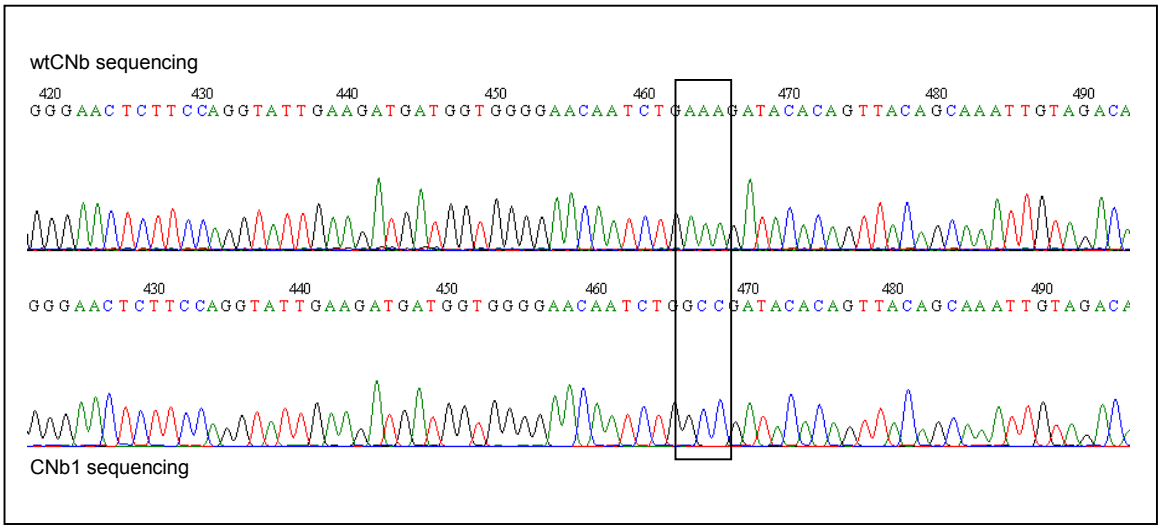


Figure 10: Example of sequencing confirming successful site directed mutagenesis. The altered amino acid is highlighted and produces the residue change K125A from wtCNb to give CNb1.

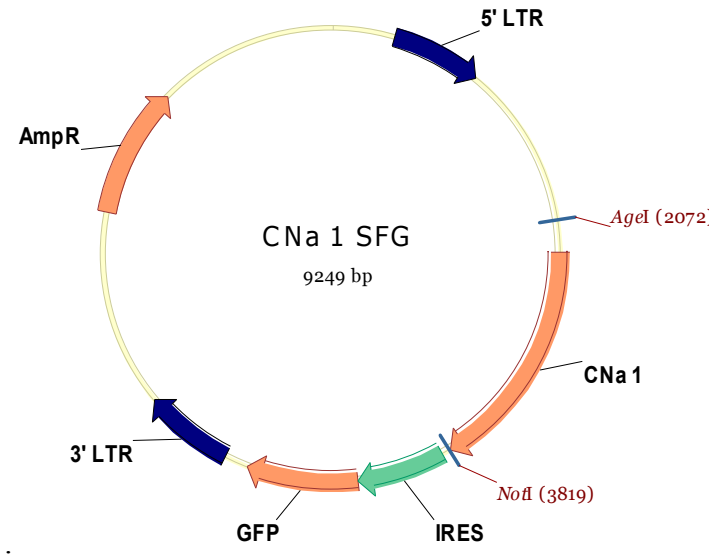


Figure 11: Diagram of SFG retroviral vector. CNa1 is followed by eGFP. LTRs, IRES and ampicillin resistance gene (Amp^R) are also indicated. AgeI/NotI sites shown were used to clone CN mutants into the SFG vector.

Mutants were subsequently cloned into the retroviral vector SFG-eGFP, upstream of an internal ribosomal entry site (IRES) allowing expression of the reporter gene eGFP. Transcription of CN in this vector is driven from viral long terminal repeats (LTRs),

with the start codon of the transgene at the site of the deleted viral *env* gene as shown in Figure 11 (Riviere*, et al* 1995). Following successful generation of this panel, the 54 mutants were screened in cell line screening assays to determine their activity in the presence of CN inhibitors.

3.3 Screening of CN mutants in cell lines assays

3.3.1 Establishing a Luciferase screening assay to identify resistant CN mutants

In order to identify CN mutants that retained the ability to dephosphorylate NFAT in the presence of CN inhibitors and may therefore confer resistance, we utilised a transient assay in 293T cells using the Dual Luciferase System (Promega). This assay measures the level of expression of two luciferase genes, Renilla luciferase derived from *R. reniformis* and Firefly luciferase derived from *Photinus pyralis*. These luciferases utilise distinct substrates for light emission, therefore sequential addition of the substrates determines expression of the two enzymes separately. In this assay, firefly luciferase (FLuc) expression was driven by an NFAT responsive promoter and Renilla luciferase (RLuc) was encoded on a constitutively active control plasmid, driven by a CMV promoter. Therefore as CN dephosphorylates NFAT and allows translocation to the nucleus, FLuc is expressed as a measure of CN activity. In contrast, RLuc expression is constitutive; its expression not affected by CN activity, and therefore can be used as an internal control for intra-assay variations in parameters such as cell density and transfection efficiency.

3.3.1.1 Optimisation of dual luciferase assay

Considerable optimisation of this assay was required. Firstly, expression of RLuc and FLuc was assessed independently and together in the absence of CN mutants to determine the limitations of the technique. Luciferase plasmids were co-transfected

into 293T cells using Lipofectamine2000, which were stimulated with PMA and ionomycin to induce cellular activation. Expression of both luciferases was assessed from untransfected or single transfected cells in the presence or absence of stimulation. As shown in Figure 12, no FLuc signal was detected in untransfected or RLuc transfected cells, and a low level of background signal from FLuc transfected cells (approx 2000 Relative Light Units (RLU)). This was increased 15-fold by stimulation of cells with PMA and ionomycin, expression which was reduced to background levels by addition of FK506. This established that the NFAT responsive FLuc plasmid is a reliable indicator of CN activity in 293T cells, and that the two substrates for RLuc and FLuc do not cross-react, as no FLuc signal is detected from an RLuc transfected sample. In addition, transfection with both Luciferases did not alter FLuc results.

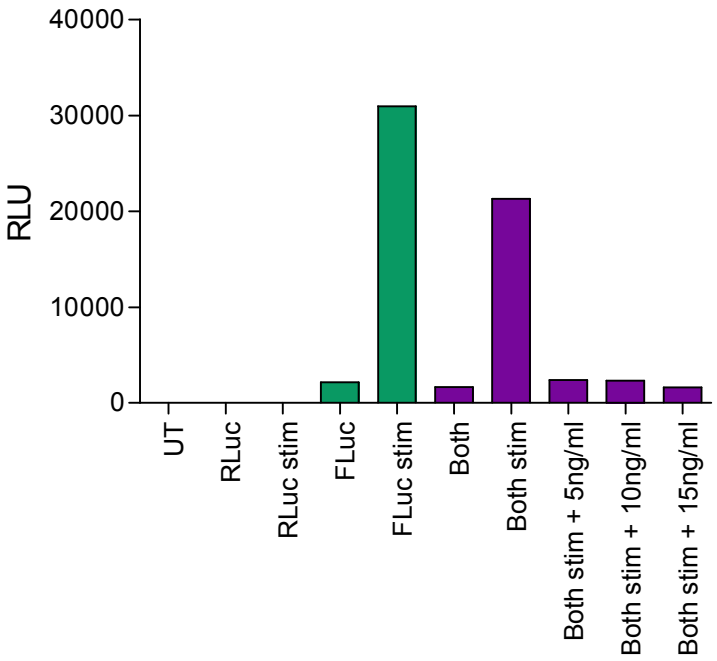


Figure 12: Expression of Firefly luciferase from transfected 293T cells. Cells were transfected either singly or with both Luciferases, and stimulated in the presence of increasing concentrations of FK506. UT: Untransfected, Stim: stimulated overnight with PMA+ionomycin, 5ng/ml: concentration of FK506 included at stimulation.

We next examined expression of RLuc in cells transfected with one or both Luciferase plasmids. Once again, no RLuc signal was detected from untransfected or FLuc transfected cells, confirming substrate specificity. However, RLuc expression from transfected cells was considerably increased with cellular stimulation, and was further amplified upon co-transfection with FLuc. Indeed, signal from RLuc in co-transfected, stimulated samples reached the upper limit of detection of the luminometer at $4x10^5$ RLU (see Figure 13a). The role of RLuc in this assay to provide a constitutively active, consistent internal control cannot be achieved if expression is affected by the activation status of the cell. We therefore substituted RLuc driven by the CMV promoter for a construct containing codon optimised RLuc under the control of the herpes simplex virus thymidine kinase (HSV-TK) promoter. This promoter lacks binding sites for many mammalian transcription factors that are present in the original CMV promoter and we hypothesised that it would therefore be less susceptible to activation in 293T cells. This plasmid was tested as previously. As shown in Figure 13b, while RLuc was expressed at much lower levels from the phRG-TK promoter, expression was unaffected by stimulation of 293T cells. Although RLuc expression was increased when co-transfected with FLuc, the signal was unaffected by stimulation or by the addition of FK506. Therefore, phRG-TK was selected as the internal control plasmid for use in the dual luciferase assay screening. FLuc expression in all subsequent experiments was normalised to RLuc expression to control for transfection efficiency, therefore data are presented as fold increase of FLuc signal.

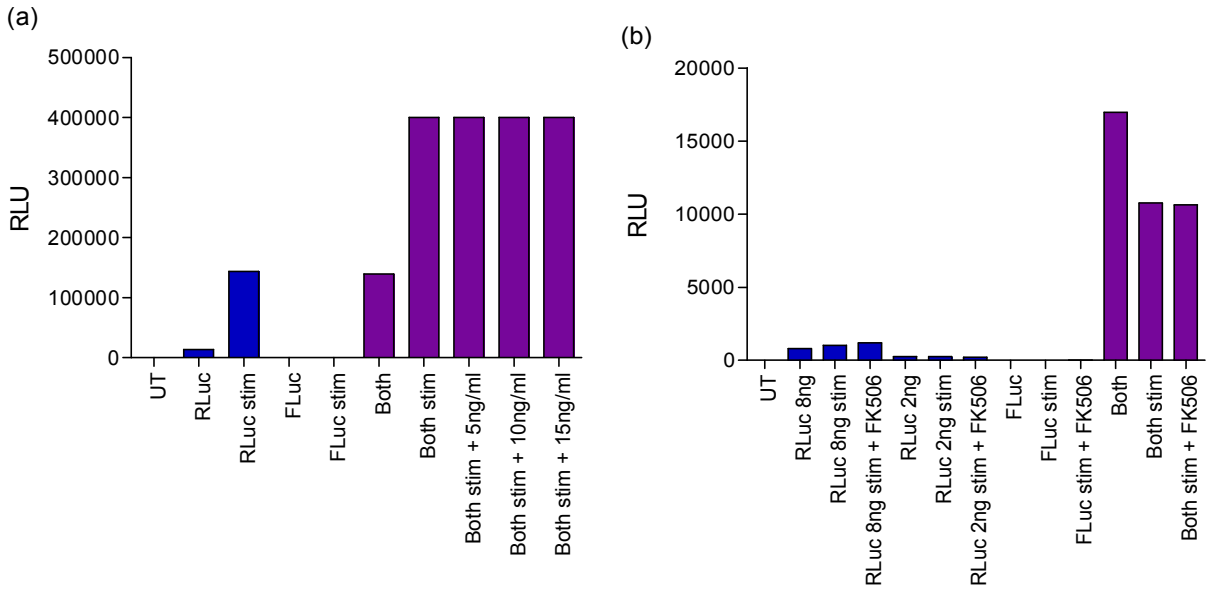


Figure 13: Renilla luciferase expression from CMV-RLuc but not phRG-TK is sensitive to cellular activation. (a) Variation in expression of original CMV-Renilla control plasmid showing effect of stimulation and co-transfection with FLuc. (b) Expression of RLuc from the indicated concentration of phRG-TK transfected alone or with FLuc, stimulated or unstimulated.

Following the selection of phRG-TK and FLuc, CNa constructs were included to produce CN mutant expressing internally controlled FLuc reporter cells to detect FK506 resistance. Transfection efficiency was assessed by monitoring expression of eGFP from the CN_eGFP plasmid. To establish the appropriate concentration of CN plasmid to confer resistance, and FLuc plasmid to report resistance, a variety of combinations were tested. Figure 14 shows that while limited resistance is seen in this experiment, the combination producing greatest FLuc expression in the presence of FK506 contains 400ng FLuc and 600ng CNa. After normalisation to RLuc signal, this sample retains a 10-fold increase in RLU expression compared to unstimulated 293T cells, therefore these concentrations were used for future experiments.

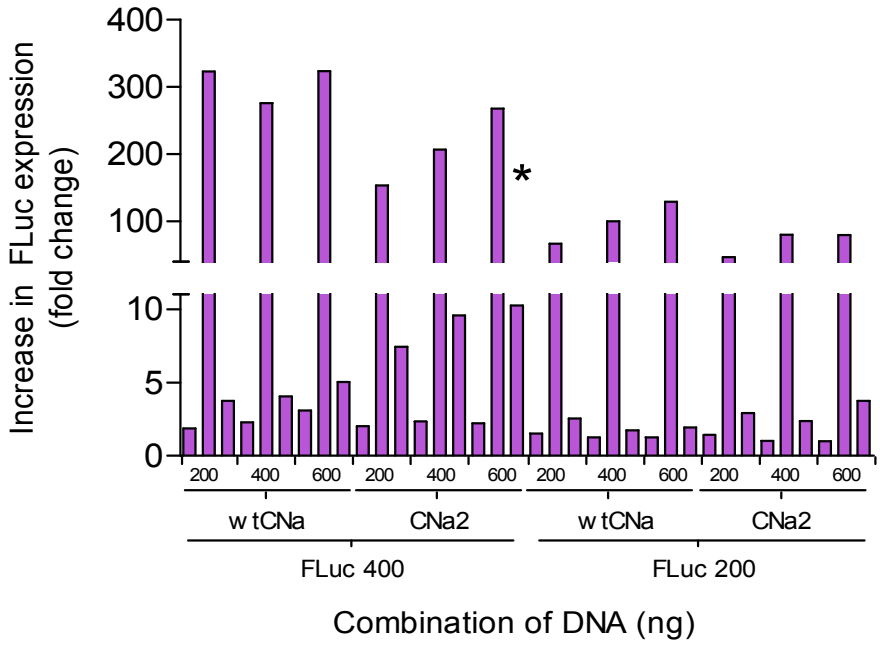


Figure 14: Determination of optimal CNa and FLuc plasmid concentration. Each set of three bars shows 1) FLuc expression in unstimulated cells 2) FLuc expression following stimulation and 3) FLuc expression following stimulation in the presence of 10ng/ml FK506. Varying amounts of CNa (wt or CNa2) and FLuc plasmids were transfected. The increase in FLuc expression compared to untransfected 293T cells is shown. The selected combination is illustrated with a star.

These parameters were subsequently used to test CNa mutants 1-8 in the presence of FK506 to determine whether this assay is sufficiently sensitive to detect resistance to this drug, as determined by continued FLuc expression in the presence of FK506. In initial experiments both CNa2 and CNa4 appeared to confer some resistance in this assay (data not shown), but the resistance observed was low when cells were assayed 24 hours after transfection. However, 293T cells transfected in this manner showed increased GFP expression at 48 hours post transfection compared with 24 hours, indicating that expression of CN mutants was also likely to be increased at this time point. Therefore, this experiment was repeated, assaying luciferase expression following stimulation at 48 hours post transfection. As shown in Figure 15, this resulted in improved detection of resistance to FK506 conferred by CNa2 and CNa4.

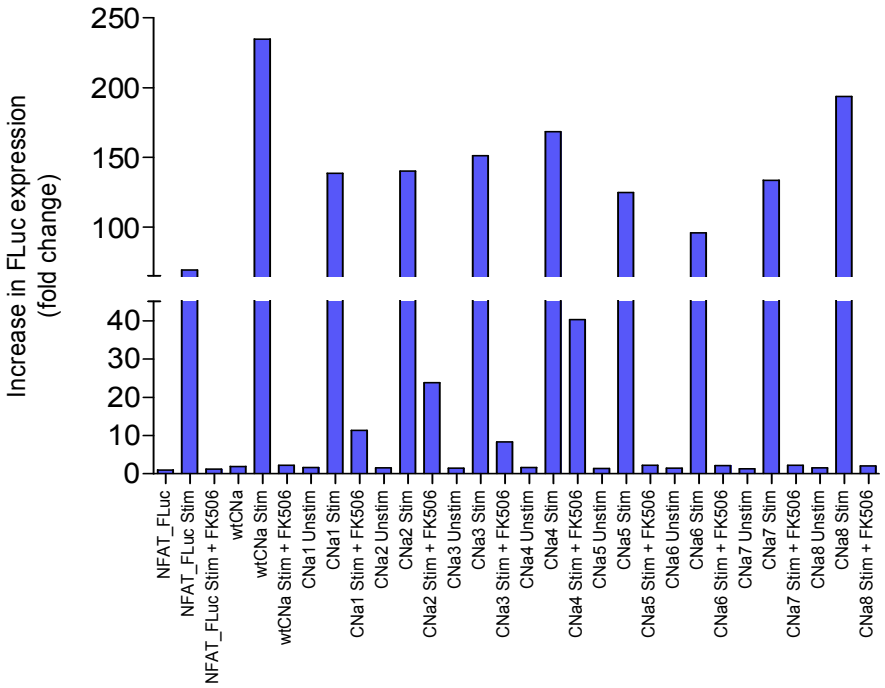


Figure 15: Luciferase assay screening of CNa mutants 1-8. Resistance to FK506 in CNa2 and CNa4 is improved by stimulation 48 hours after transfection.

In order to determine the reliability of the screening assay, this experiment was repeated twice. Similar results were obtained in each experiment, confirming that this assay is reliable and can be used to screen the panel of mutants, identifying those that confer resistance and excluding those that do not (see Figure 16a). To ease comparison between different mutants, resistance was then expressed as the percent activity retained in the presence of FK506, compared to the activity of that sample in the absence of FK506 (see Figure 16b).

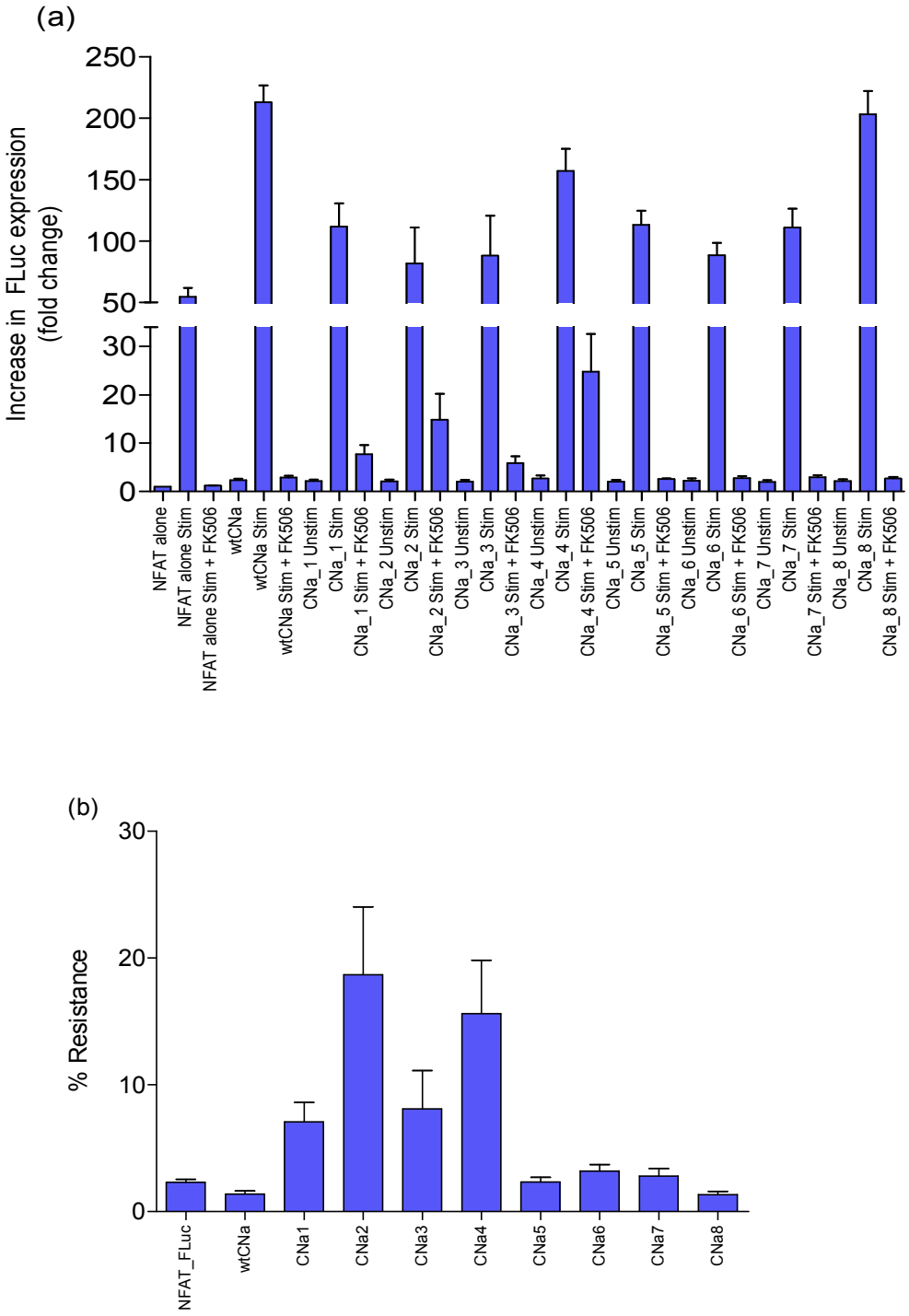


Figure 16: Dual luciferase screening is reliable in multiple assays. a) Screening of CNa mutants 1-8. Resistance to FK506 was detected in samples transfected with mutants 2 and 4 in all assays performed. b) Resistance for each mutant expressed as the percent signal in the presence of FK506 compared to the stimulated signal for that sample. Mean + SEM of 3 experiments shown.

3.3.2 Screening of CNa and CNb mutants in Luciferase assay

Following optimization of this screening assay so that it performs adequately to differentiate those mutants that confer some resistance from those that do not, all 54 CNa and CNb mutants were screened as described above to determine resistance to 10ng/ml FK506 or 200ng/ml CsA. Figure 17 shows the percent resistance conferred by CN mutants to FK506 and CsA, defined as the percent FLuc signal (normalised for RLuc) in the presence of CN inhibitor compared to 293T cells transfected with the same mutants stimulated in the absence of CN inhibitor. This guided the selection of 6 CNa mutants that conferred resistance to FK506 (CNa2, 4, 9, 12, 15 and 17) and 5 resistant to CsA (CNa18, 19, 20, 21 and 22) and 6 CNb mutants, one resistant to FK506 only (CNb12), one resistant to CsA only (CNb26) and four conferring resistance to both (CNb21, 22, 23 and 30). This threshold for selection (15% resistance) was determined by comparison of CNa mutants 1-8 to an initial screen in the subsequent assay utilising Jurkat cells as described below. These 17 CN mutants were then pursued in further testing.

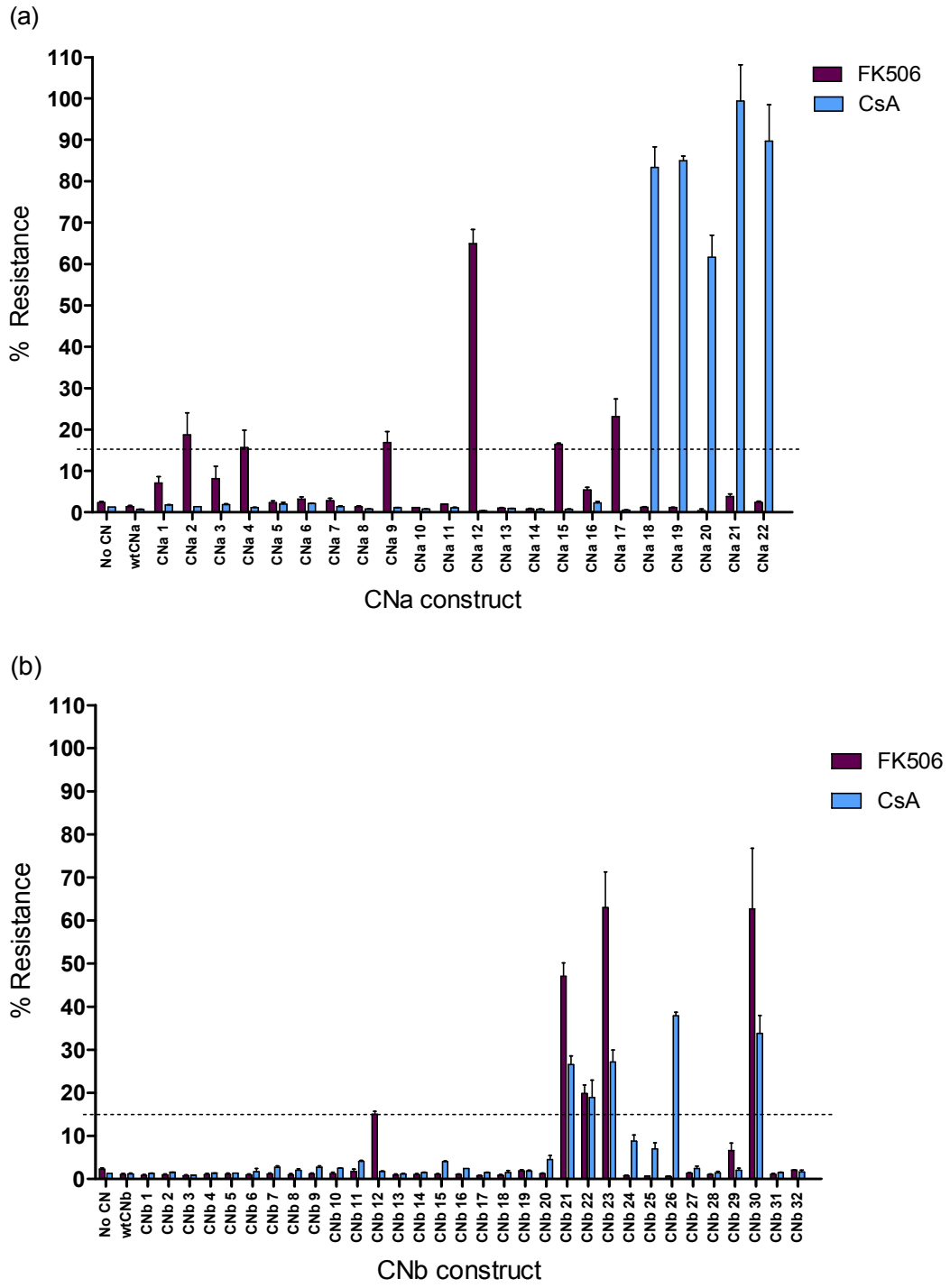


Figure 17: Transfection of 293T cells with CN mutants allows luciferase expression in the presence of CN inhibitors. Screening of (a) CNa mutants and (b) CNb mutants for resistance to 10ng/ml FK506 (purple bars) and 200ng/ml CsA (blue bars). Resistance was determined by comparing luciferase expression upon stimulation in the presence of CN inhibitors to luciferase expression upon stimulation in the absence of CN inhibitors. Mean and SEM of 3 experiments shown.

3.3.3 Development of Jurkat IL-2 secretion assay

To assess the capability of the selected mutants to confer resistance to CN inhibitors in a biologically relevant model, cytokine secretion by the Jurkat cell line was assessed. Jurkats are a human T cell line, which secrete IL-2 in response to stimulation with mitogens such as PMA and ionomycin. However, IL-2 secretion in response to such stimulation is prevented by the addition of FK506 or CsA, thus allowing detection of resistance.

3.3.3.1 Source of Jurkat cells and detection of IL-2 secretion

Although Jurkat cells are reported to be capable of high IL-2 secretion, significant batch-to-batch variation has been observed in this regard. Therefore, we obtained Jurkat cells from several sources and stimulated these to determine which would be most appropriate for use in further studies. IL-2 secretion from Jurkats was measured by ELISA 24 hours after stimulation with 20ng/ml PMA and 1μg/ml ionomycin, and it was established that of these 5 cultures, batches 2 and 4 secreted no IL-2, batch 1 secreted negligible amounts that increased slightly upon stimulation, batch 5 secreted no IL-2 without stimulation and approximately 1000pg/ml with stimulation, however batch 3 secreted no IL-2 without stimulation but secreted approximately 5000pg/ml following stimulation with mitogens (see Figure 18). Therefore, batch three (obtained from Dr Clio Rooney, Baylor College of Medicine, TX, USA) was selected for further use.

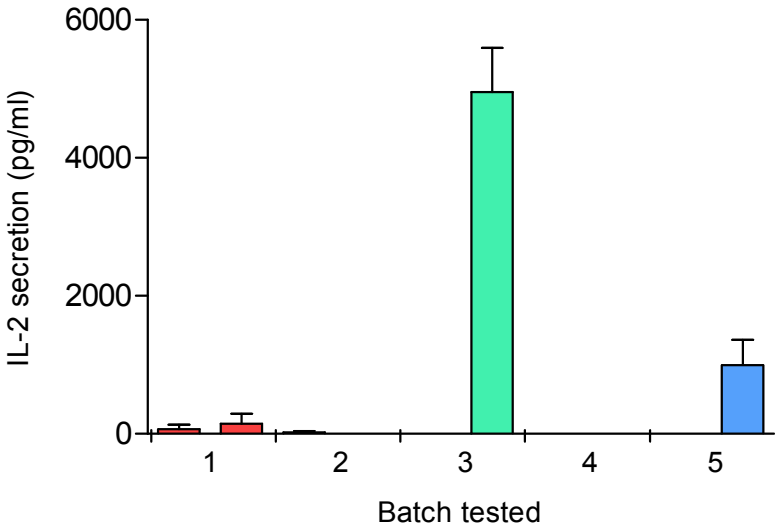


Figure 18: Variable IL-2 secretion from different batches of Jurkat cells. Jurkat cells from five different sources were stimulated with PMA and ionomycin, IL-2 secretion by each batch before and after stimulation were measured by ELISA. Mean and SEM of two experiments. Batch 1 was previously used in our laboratory, batch 2 was a gift from Dr. W. Qasim, ICH, batches 3 and 4 were a kind gift from Dr Clio Rooney, Baylor College of Medicine, and batch 5 was purchased from the DSMZ cell bank.

As a result of high IL-2 secretion by the selected batch of Jurkats, it was necessary to dilute the samples as those tested initially were above the linear range of the assay. Therefore, supernatants from two experiments were assayed either neat and diluted 1/20, or diluted 1/3 and diluted 1/21 respectively. This established that considerable dilution of Jurkat supernatants from this batch was necessary to obtain accurate results (see Figure 19). Stimulated Jurkat cells from batch 3 secreted up to 15000pg/ml IL-2, which was accurately measured when tested at a dilution of approximately 1/20. Results from these samples remained in the upper portion of the linear range therefore subsequent ELISAs were performed using samples diluted 1/40, which consistently produced results within the range of the assay (between approximately 50 and 1000pg/ml IL-2). It was also confirmed that this dilution did not increase background and that addition of 10ng/ml FK506 to the cells at stimulation abrogated secretion of IL-2 (data not shown).

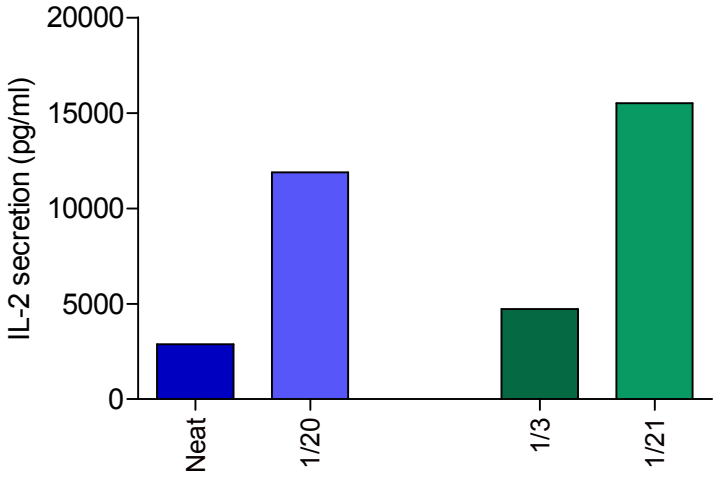


Figure 19: Dilution of Jurkat supernatants allows accurate measurement of IL-2 concentration. Supernatant harvested from stimulated Jurkats were tested by ELISA both neat and in dilutions. Samples from two separate experiments were assayed at low or no dilution and at 1/20 or 1/21 dilution. Diluted samples were in the linear range of the assay and therefore gave an accurate result.

3.3.3.2 Transduction of Jurkat cells

RD114 pseudotyped retrovirus was generated by triple transfection of 293T cells with SFG_CN_eGFP, GagPol (PeqPam) and Env (RDF) plasmids. Transient supernatants were harvested at 48 and 72 hours, combined and snap frozen before transduction of Jurkat cells using Retronectin and spinfection. This method achieved good transduction efficiencies as determined flow cytometrically by eGFP expression. Further, as shown in Figure 20, transgene expression was stable for at least 11 weeks in culture.

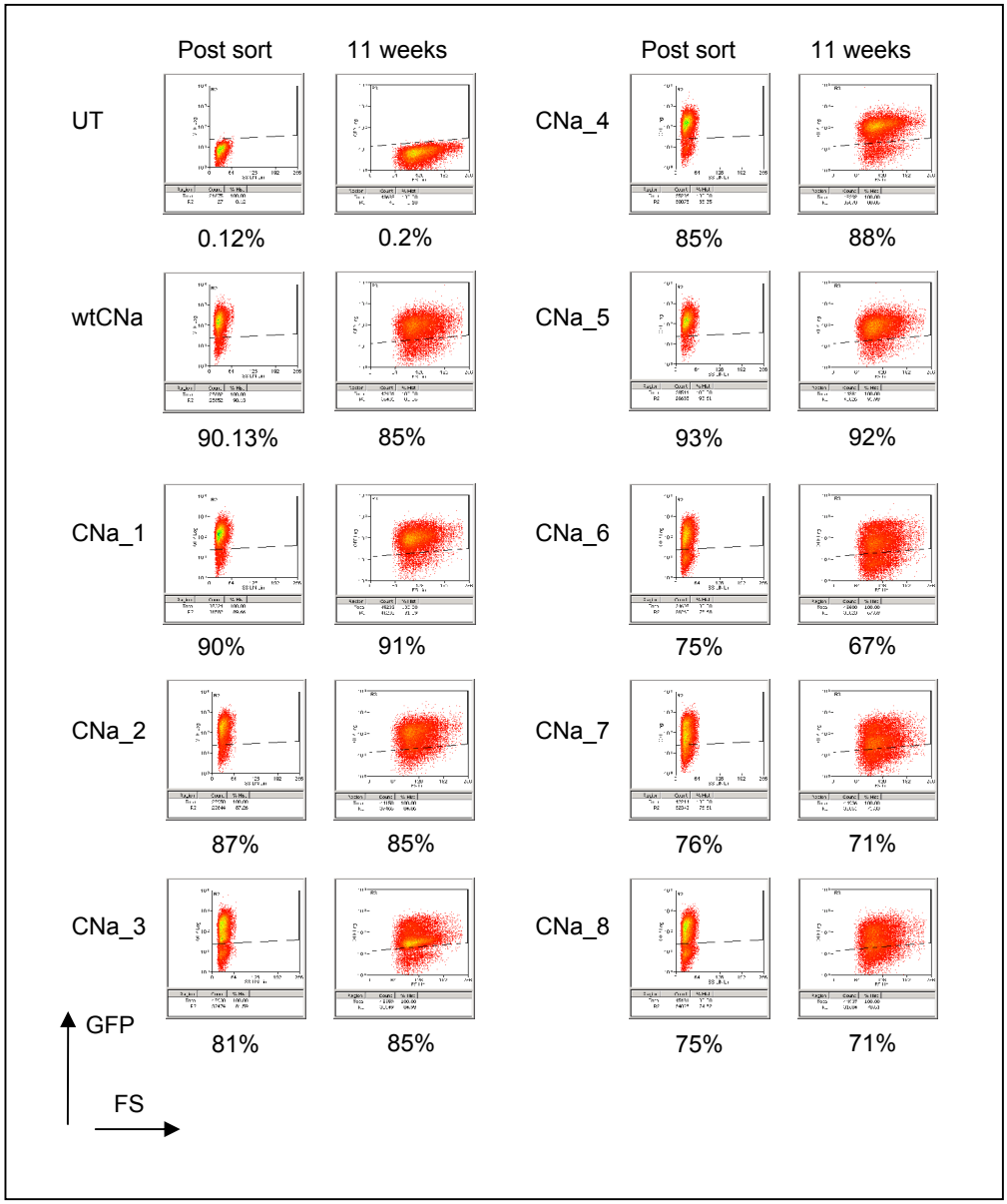


Figure 20: Expression of CN mutants remains stable long term *in vitro*. Jurkat cells were FACS sorted and maintained in culture for 11 weeks to ensure continuous transgene expression. GFP expression as determined by flow cytometry 1 week post sort is displayed on the left, along with the corresponding data following 11 weeks in continuous culture on the right.

3.3.3.3 Correlation of Jurkat IL-2 ELISA with 293T luciferase assay

Following establishment of the Jurkat model and confirmation that efficient transduction of these cells can be achieved using SFG retrovirus containing CN mutants, initial testing of resistance was undertaken to compare the performance of Luciferase screening with the Jurkat assay. To determine the appropriate cut-off for the Luciferase assay to select which mutants to pursue, RV was generated from CNa mutants 1-8 and transduced Jurkats were assessed for their ability to secrete IL-2 in the presence of FK506. Figure 21 shows that in the presence of 10ng/ml FK506, CNa2 transduced cells secreted IL-2 at 34% of the level secreted by UT Jurkats in the absence of IS. In contrast, CNa1 transduction allowed secretion of IL-2 at 9% of the level secreted by untransduced Jurkat cells. In corresponding luciferase assays in 293T cells, CNa1 gave a mean 7% resistance to FK506, whereas CNa2 gave a mean 19% resistance. To rationalise the number of mutants tested in the Jurkat system, a cut-off of 15% resistance in FLuc expression was set, which would select CNa2 but not CNa1 for further study. This threshold was applied to all other CN mutants and led to selection of the 17 mutants described in section 3.3.2.

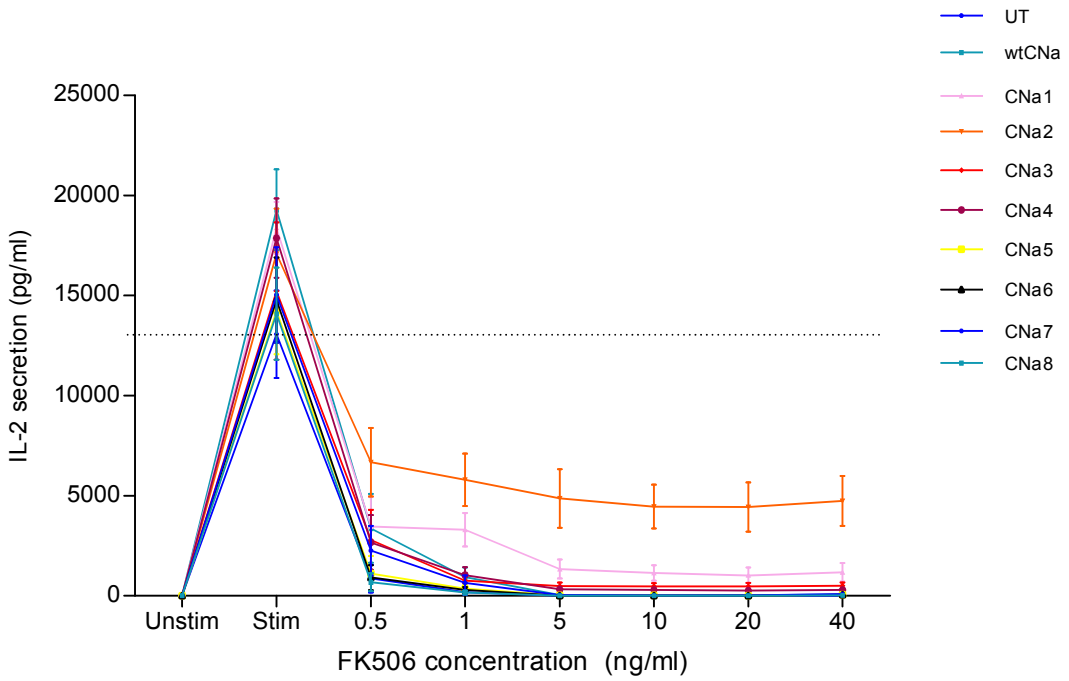


Figure 21: Transduction with CN mutants allows IL-2 secretion from Jurkat cells in the presence of FK506. IL-2 secretion from unstimulated Jurkat cells (unstim), cells stimulated for 24 hours with 20ng/ml PMA and 1µg/ml ionomycin (stim), or cells stimulated in this manner with addition of the indicated concentration of FK506 (0.5-40ng/ml). Cells transduced with CNa2 (34%) and CNa1 (9%) were able to secrete IL-2 despite increasing concentrations of FK506. Dotted line indicated secretion of IL-2 by UT Jurkat cells without CN inhibitors (100%). Mean and SEM of three experiments are shown.

3.3.4. Identification of resistant CN mutants in IL-2 secretion assay

Following optimisation of the assay, Jurkat cells were transduced with all the selected mutants and transduction efficiency was assessed. Where transduction was below 80%, Jurkats were sorted to select GFP expressing cells, however where greater than 80% GFP expression was achieved cultures were not sorted. These cells were then tested as described above, to assess their ability to secrete IL-2 in the presence of either FK506 or CsA, based on the resistance profile identified by Luciferase screening.

Considerable variation in the amount of IL-2 secreted between assays was observed, compounding direct comparison. To account for inter-assay variation and facilitate

accurate interpretation of results, IL-2 secretion is calculated compared to the stimulated UT sample in the absence of CN inhibitors for each experiment. The resulting percent IL-2 secretion was combined to give a mean percent secretion compared to this standard level.

Figure 22(a) shows IL-2 secretion from CNa transduced Jurkats in the presence of FK506. IL-2 secretion by UT and wtCNa transduced Jurkats is abrogated by FK506 at levels as low as 0.5ng/ml. In general, there was a reasonable correlation with the 293T luciferase assay, with several of the mutants identified also showing resistance in the Jurkat IL-2 secretion assay. Mutant CNa12, which gave the highest resistance to FK506 in the Luciferase assay, retains the ability to secrete IL-2 up to the highest concentration tested (40ng/ml), which is significantly higher than the therapeutic range (7-12ng/ml). Compared to IL-2 secretion by stimulated UT Jurkats with no FK506, CNa12 transduced Jurkats retained a mean 84% IL-2 secretion in the presence of 10ng/ml FK506. CNa2 also retains some activity in the presence of 10ng/ml FK506, secreting IL-2 at a mean of 35% of the level secreted by untransduced Jurkat cells. Mutants CNa 15 and 17 continue to secrete IL-2 in the presence of 0.5 or 1ng/ml FK506, however this secretion drops at concentrations of 5ng/ml or higher. CNa4, 9 and 12 did not confer resistance to FK506.

As shown in Figure 22(b), CNa mutants CNa18, 19, 21 and 22 secrete high levels of IL-2 throughout the therapeutic range of CsA (100-250ng/ml) and in the presence of supratherapeutic concentrations up to 800ng/ml. When compared to secretion by UT Jurkats without CsA, these mutants secrete between 171% (CNa18) and 259% (CNa22) IL-2 at 200ng/ml CsA. CNa20 conferred 40% resistance to CsA. It is notable that in this assay, those cells that were transduced with CNa mutants 18, 19, 21 and 22 secreted an increased level of IL-2 upon stimulation compared to UT Jurkats. This effect was not observed with wtCNa or other mutants. This could be a consequence of the increased level of CN in the cell activating more NFAT and therefore leading to more IL-2 secretion, however this increase was not observed in cells transduced with wtCNa. It is possible that alteration of residue V314, which is common to those four mutants, affects either the activity or the stability of the CN molecule, allowing hyper-

activity. This was a consideration when selecting the appropriate mutants for further study.

Jurkat cells transduced with CNb mutants were also assessed for secretion of IL-2 in the presence of CN inhibitors. As shown in Figure 23, mutant CNb30 provided the highest resistance to both CN inhibitors, secreting 47% IL-2 in the presence of10ng/ml FK506 and 26% with 200ng/ml CsA. CNb21 also retained some activity, with 26% resistance to FK506 and 8% to CsA and CNb23 retained 24% IL-2 secretion with FK506 and 9% with CsA. In this assay, CNb12 and 22 did not confer significant resistance to FK506 and CNb22 and 26 showed no resistance to CsA. Interestingly, all CNb mutants that conferred resistance contained a 2AA insertion at position K125, despite some other mutants being identified in the Luciferase screening assay. Furthermore, transduction with CNb mutants did not result in increased IL-2 secretion upon stimulation compared to UT Jurkats.

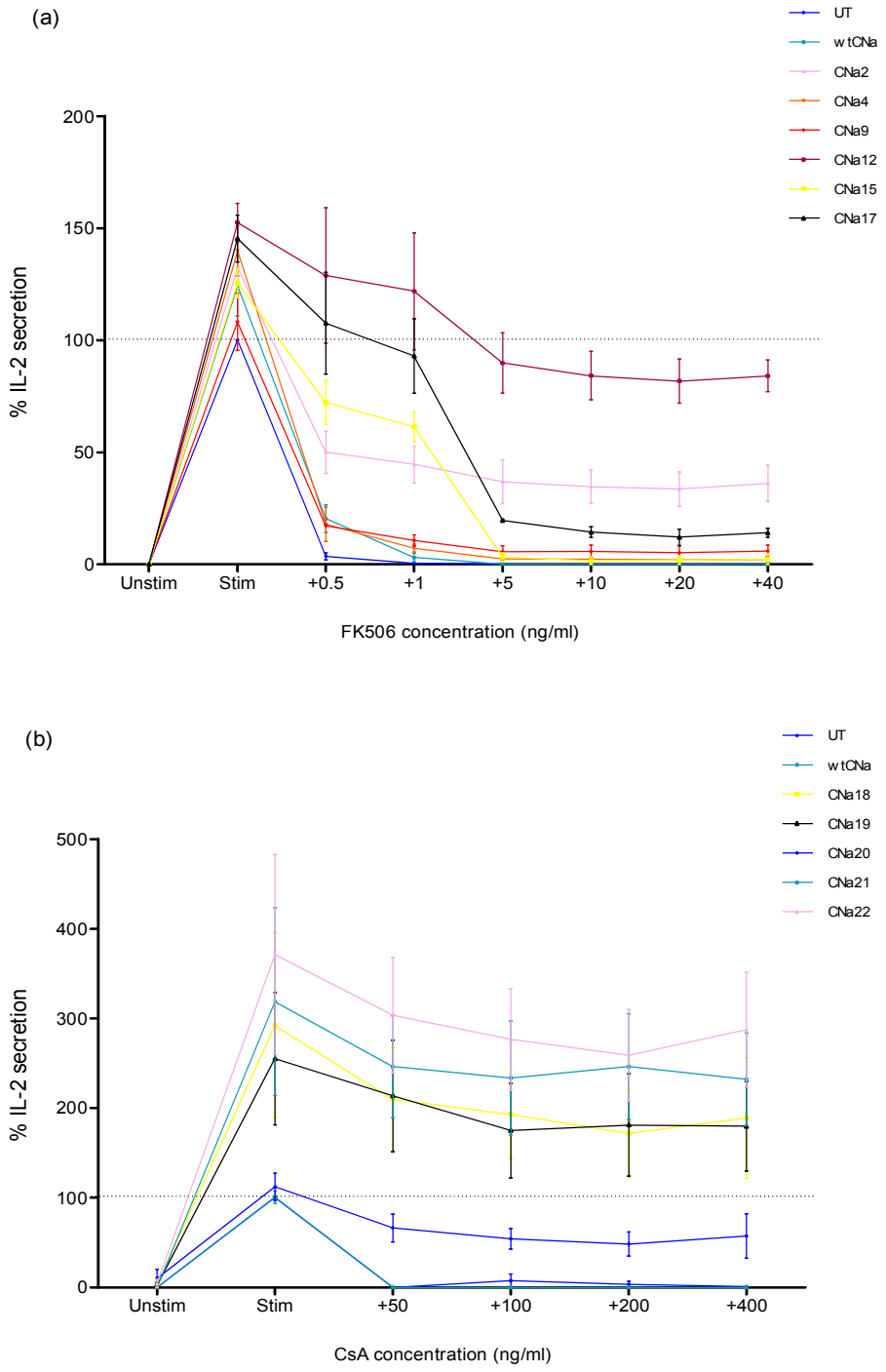


Figure 22: Transduction of Jurkat cells with CNa mutants allows IL-2 secretion in the presence of FK506 (a) or CsA (b). Mean and SEM of three experiments shown. Dotted line indicates IL-2 secretion by UT Jurkat cells stimulated without CN inhibitors (100%).

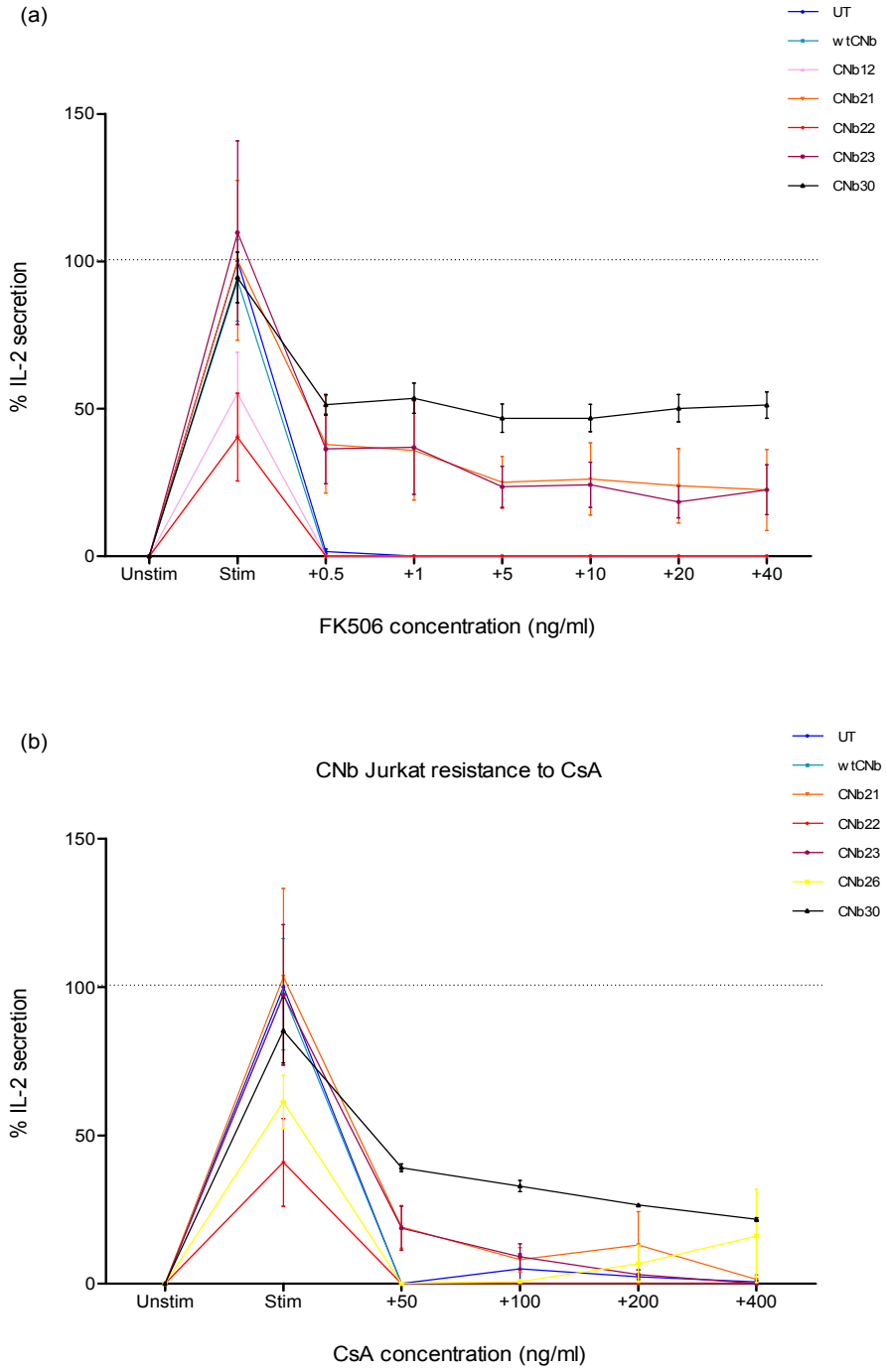


Figure 23: IL-2 secretion by CNb transduced Jurkat cells in the presence of increasing concentrations of FK506 (a) or CsA (b). Mean and SEM of three experiments shown. Dotted line indicates IL-2 secretion by UT Jurkat cells stimulated without CN inhibitors (100%).

3.3.5. Analysis of CN expression following transduction

Some variation was observed in the mean fluorescence intensity (MFI) of GFP expression between samples transduced with different mutants, therefore differences in CN expression were also likely. To exclude the possibility that differences in the resistance profile to FK506/CsA were a result of differences in CN expression, we analysed expression of CN directly using Western blotting. Jurkat cells transduced with retroviral supernatants generated during different batches of production were compared to establish the variation in CNa expression between individual mutants or RV batches. In particular, we looked at expression of those mutants that gave resistance, to determine whether these expressed the highest levels of CNa.

Samples were tested from all cultures transduced with virus made in two different batches: Retroviral supernatants for CNa1-8 were generated first, and supernatants for CNa9, 12, 15 and 17 were generated later, and had a higher MFI when GFP expression was assessed by flow cytometry, therefore these two batches were examined for CNa expression. Lysate was generated from $2x10^6$ transduced or untransduced Jurkat cells, and 40µl loaded per sample. Membranes were cut into sections based on the expected size of CNa and β-actin and each portion probed with the appropriate antibody. Bands were quantified and expression of CNa was normalised according to the β-actin expression for each sample. Expression of CNa in transduced cells was compared to that in untransduced control samples. As shown in Figure 24, while β-actin expression was similar between lysates, the expression of CNa mutants, though always higher than untransduced samples, varied widely.

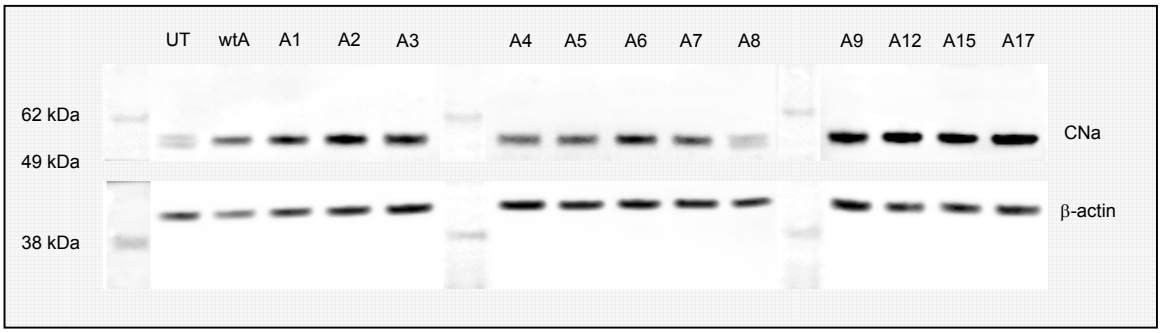


Figure 24: Jurkat cells transduced with virus from different batches show variation in CNa expression. Expression of β-actin is shown to control for protein loading. RV containing CNa1-8 was generated together; RV containing CNa9, 12, 15 and 17 was generated at a separate time point. Quantification of CN and β-actin expression is shown in table 5.

Table 5: Quantification of protein bands identified by Western blot. CN expression is adjusted to account for variation in protein loading established by quantification of β-actin. UT in yellow, RV batch 1 highlighted in blue (average CNa increase 2.5), RV batch 2 highlighted in purple (average CNa increase 5.4).

Sample	Actin expression	Relative Actin	CNa expression	Adjusted CNa	Relative CNa
UT	256573	1.39	37394	26922	1.00
wtCNa	184720	1.00	88932	88932	3.30
CNa1	291505	1.58	140428	88986	3.31
CNa2	364950	1.98	202181	102334	3.80
CNa3	475205	2.57	172876	67200	2.50
CNa4	489258	2.65	115091	43453	1.61
CNa5	400919	2.17	125468	57808	2.15
CNa6	410236	2.22	155501	70019	2.60
CNa7	382215	2.07	107311	51862	1.93
CNa8	299687	1.62	54663	33693	1.25
CNa9	446198	2.42	233043	96477	3.58
CNa12	326098	1.77	310761	176032	6.54
CNa15	358333	1.94	265704	136970	5.09
CNa17	406611	2.20	369523	167871	6.24

Table 5 shows the relative expression of CNa mutants compared to UT Jurkat cells, normalised to β-actin expression. These data establish that there is considerable variation in the expression of CN both within and between batches of RV. RV containing CN mutants 1-8 was generated at the same time point, however CN expression varies between cells transduced with the different retroviral supernatants. For example, cells expressing mutant CNa4 show a 1.6 fold increase in CNa expression compared to UT Jurkats, whereas those transduced with CNa2 show a 3.8 fold increase. CNa8 appears to be unstable as there is very little increase in expression from UT, despite comparable levels of GFP. This variation is increased further between batches of virus made at different times, with expression of CN from Jurkats transduced with mutants CNa9, 12, 15 or 17 all showing further increased expression of between 5 and 6.5 fold compared to UT Jurkat cells. Overall, average CN expression from cells transduced with RV batch 1 was 2.5 fold above UT, compared to a 5.4 fold increase in cells transduced with RV from batch 2. Therefore, data concerning relative resistance to CN inhibitors generated from these cells are questionable and might be attributed to differences in CN expression rather than mutant activity.

3.3.6. FACS sorting of transduced Jurkat cells and re-analysis

In order to control for the variability in CN mutant expression observed above, all cultures were sorted by fluorescence activated cell sorting (FACS) to comparable levels of GFP expression to reduce CN variation. Since expression of CN mutants occurs from the same RNA transcript as GFP, normalising for GFP expression should ensure that differences observed in resistance to CN inhibitors reflect true differences in the level of activity retained by CN mutants rather than differences in the amount of CN expressed. The mean fluorescence intensity (MFI) of GFP expression following FACS sorting of retrovirally transduced Jurkat cells is shown in Table 6. It can be seen that CNb mutants were sorted to a higher level of GFP expression (mean MFI 570) than CNa mutants (mean MFI 75), reflecting that CNb is a smaller transgene, therefore

expression of GFP from an IRES following CNb is likely to be brighter compared to expression following the larger CNa

Table 6: Mean fluorescence intensity of GFP comparable following FACS sorting.

Construct	MFI
UT	-
wtCNa	76
CNa2	89
CNa4	78
CNa9	87
CNa12	83
CNa15	88
CNa17	80
CNa18	66
CNa19	60
CNa20	66
CNa21	72
CNa22	59
wtCNb	487
CNb12	556
CNb21	601
CNb22	600
CNb23	574
CNb26	577
CNb30	593

Following FACS sorting, transduced Jurkat cultures were again examined by Western blot to confirm comparable expression of CNa/CNb (Figure 25 and Figure 26). These data establish that variation in CN expression between cultures is reduced following sorting of Jurkats to similar GFP level. Quantification of β-actin and CNa is presented in Table 7, showing increase in CNa expression of between 2.7 and 13.1 fold (CNa4 and CNa20 respectively) by transduced compared to UT Jurkats. This range of CNa expression is larger than anticipated after FACS sorting of Jurkat cells, however with the exception of CNa4, 9 and CNa20, all other samples show consistent increase in CNa expression of 5.2 to 8.6 fold compared to UT cells. It remains possible that the

mutations introduced into the CN molecule affect the stability of these mutants and that the observed differences in expression reflect reduced/increased half-life in the cells, rather than variable transduction or sorting. As our aim is to identify those mutants that are able to confer the highest resistance to FK506/CsA, a mutant with reduced stability would be undesirable for this purpose although it may render cells resistant if expressed at a higher level. Therefore, screening was continued with no further attempts to normalise CN expression in outlying cultures.

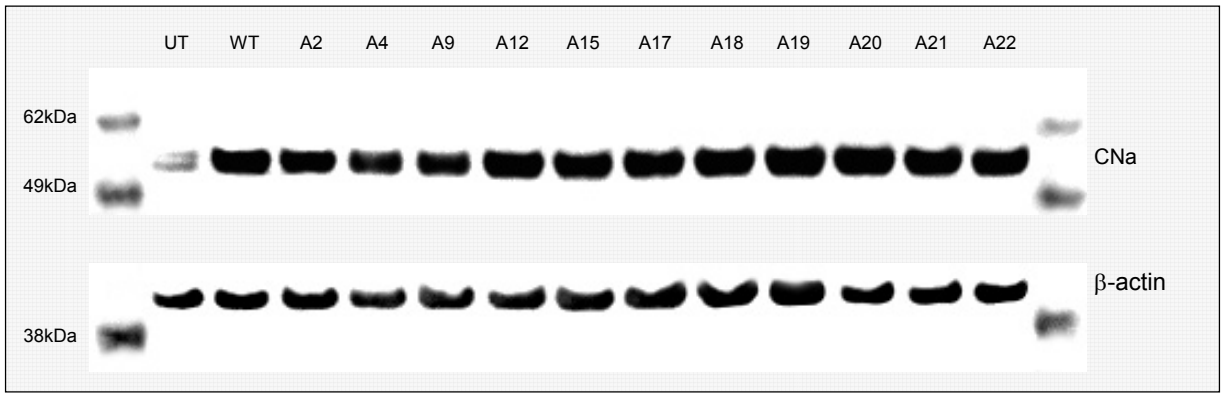


Figure 25: CNa expression between transduced Jurkat cells is comparable following FACS sorting. Untransduced Jurkat cells (UT) are compared to cells transduced with wild type CNa (WT) or CNa mutants (A2-A22). β-actin is shown as an internal control. Quantification of CNa bands, normalised for variation in protein loading, shows an 8 fold increase of CNa expression when transduced with wild type. Cultures transduced with mutant CNas show variable increase of expression, from 2.7 fold (A4) to 13 fold (A20) increase compared to untransduced Jurkats. For band quantification see Table 7.

Table 7: Quantification of CNa expression from sorted Jurkat cells. Expression of CNa is adjusted for variation in protein loading as determined by β-actin staining. Outlying cultures are shaded.

Sample	Actin	Relative actin	CNa	Adjusted CNa	Relative increase
UT	476029	1.00	31869	31869	1.00
wtCNa	566859	1.19	320298	269158	8.44
CNa2	584732	1.23	204542	166294	5.23
CNa4	546819	1.15	98780	858956	2.70
CNa9	584359	1.23	110754	90044	2.83
CNa12	556635	1.17	308563	263729	8.28
CNa15	595646	1.25	219259	175407	5.50
CNa17	684073	1.44	274984	190961	6.00
CNa18	718771	1.51	322832	213796	6.71
CNa19	794290	1.67	374349	224161	7.04
CNa20	537082	1.13	472480	418124	13.14
CNa21	578963	1.22	335523	275019	8.66
CNa22	619985	1.30	257329	197945	6.20

Expression of CNb in transduced Jurkat cells is shown in Figure 26, and quantification of these bands in Table 8. Jurkat cells transduced with CNb and sorted to the same level of GFP expression show a consistent increase in CNb expression of between 1.8 and 3.8 fold compared to UT Jurkat cells (wtCNb and CNb21 respectively). Interestingly, while the MFI of cultures transduced with CNb mutants was considerably higher than those transduced with CNa mutants, a more modest increase in CNb than CNa expression was generally observed. This may reflect the level of wtCNa compared CNb expressed in untransduced Jurkats.

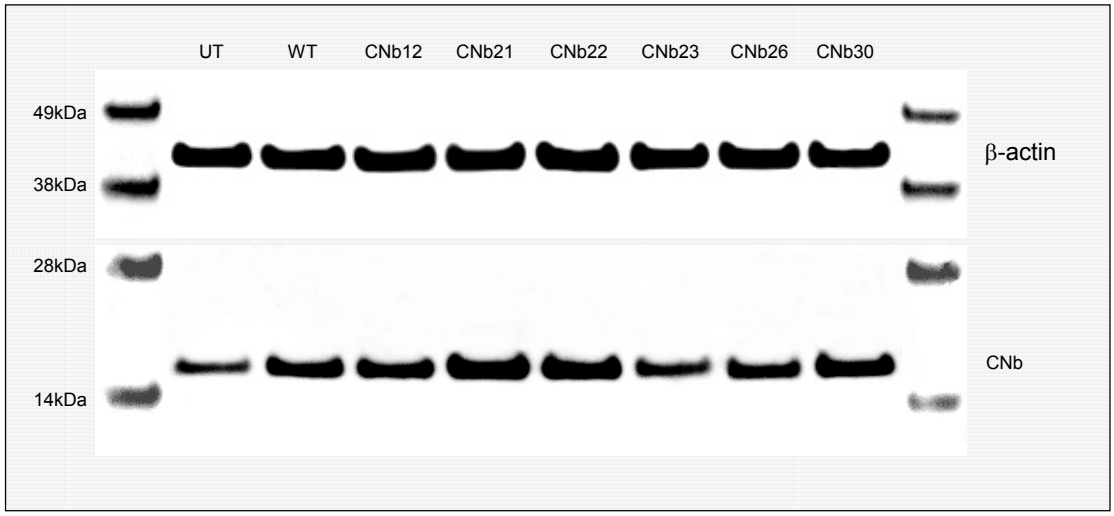


Figure 26: CNb expression between transduced Jurkat cells is comparable following FACS sorting. Untransduced Jurkat cells (UT) are compared to cells transduced with wild type CNb (WT) or CNb mutants (CNb12-30). β-actin is shown as internal control. Quantification of CNb bands, normalized for variation in protein loading, shows a 1.8 fold increase of CNb expression when transduced with wild type. Cultures transduced with mutant CNbs show variable increase of expression, from 1.96 fold (CNb23) to 3.75 fold (CNb21) increase compared to untransduced Jurkats.

Table 8: Quantification of CNb expression from sorted Jurkat cells. Expression of CNb is adjusted for variation in protein loading as determined by β-actin staining.

Sample	Actin	Relative actin	CNb	Adjusted CNb	Relative increase
UT	96178	1.25	1381600	1105280	1.00
wtCNb	94041	1.22	2403819	1970343	1.78
CNb12	80203	1.04	2708518	2604344	2.35
CNb21	77013	1.00	4147483	4147483	3.75
CNb22	105693	1.37	3818658	2787342	2.52
CNb23	101715	1.32	2857820	2165015	1.96
CNb26	107522	1.40	3025538	2161099	1.96
CNb30	92351	1.20	3414232	2845193	2.57

After standardization of CN expression between transduced Jurkat cells by sorting to consistent GFP levels, the resistance conferred by CN mutants was re-tested. Once again Jurkats were stimulated with mitogens in the presence of increasing concentrations of CN inhibitors and their ability to secrete IL-2 was determined.

Figure 27 illustrates that CNa12 again provides the highest resistance to 10ng/ml FK506, with 85% secretion of IL-2 compared to UT cells in the absence of CN inhibitors. CNa2 also confers resistance, with 68% IL-2 secretion. In the presence of CsA, those four mutants that secreted the highest IL-2 before sorting continued to do so in this assay, with CNa22 secreting 368% IL-2 compared to UT Jurkats (without CN inhibitors), CNa19 secreting 288%, and CNa18 and 21 secreting intermediate levels.

In addition, the previously noted increase in secretion of IL-2 from mutants CNa18, 19, 21 and 22 is observed from other constructs following FACS sorting. Indeed, increased IL-2 secretion of varying degrees occurs from all CNa transduced cultures including wtCNa in this experiment. It is therefore likely that this is a general effect of increased CN expression rather than hyperactivity of particular mutants. During FACS sorting cells expressing high levels of GFP, and therefore CNa, were selected. For example, prior to sorting the wtCNa transduced culture expressed 3.3 fold more CNa than UT, however after sorting the cells expressed 8.4 fold more CNa. After FACS selection, CNa transduced cells secreted 280-380% of the amount of IL-2 secreted by untransduced Jurkats in the absence of CN inhibitors. However, it is interesting to note that despite this increase, cells transduced with wtCNa are not rendered resistant to FK506 or CsA, indicating that in our assay simple over-expression of CNa is not sufficient to enable IL-2 secretion in the presence of even low levels of either drug. This is in contrast to some previous studies which find that over-expression of wtCNa results in some resistance to CsA in a transfected Jurkat cell system, increasing the IC_{50} by 2.5-5 fold (Zhu, *et al* 1996). It is possible that our assay was not sufficiently sensitive to detect small changes as we have focused on resistance to therapeutic levels of CN inhibitors.

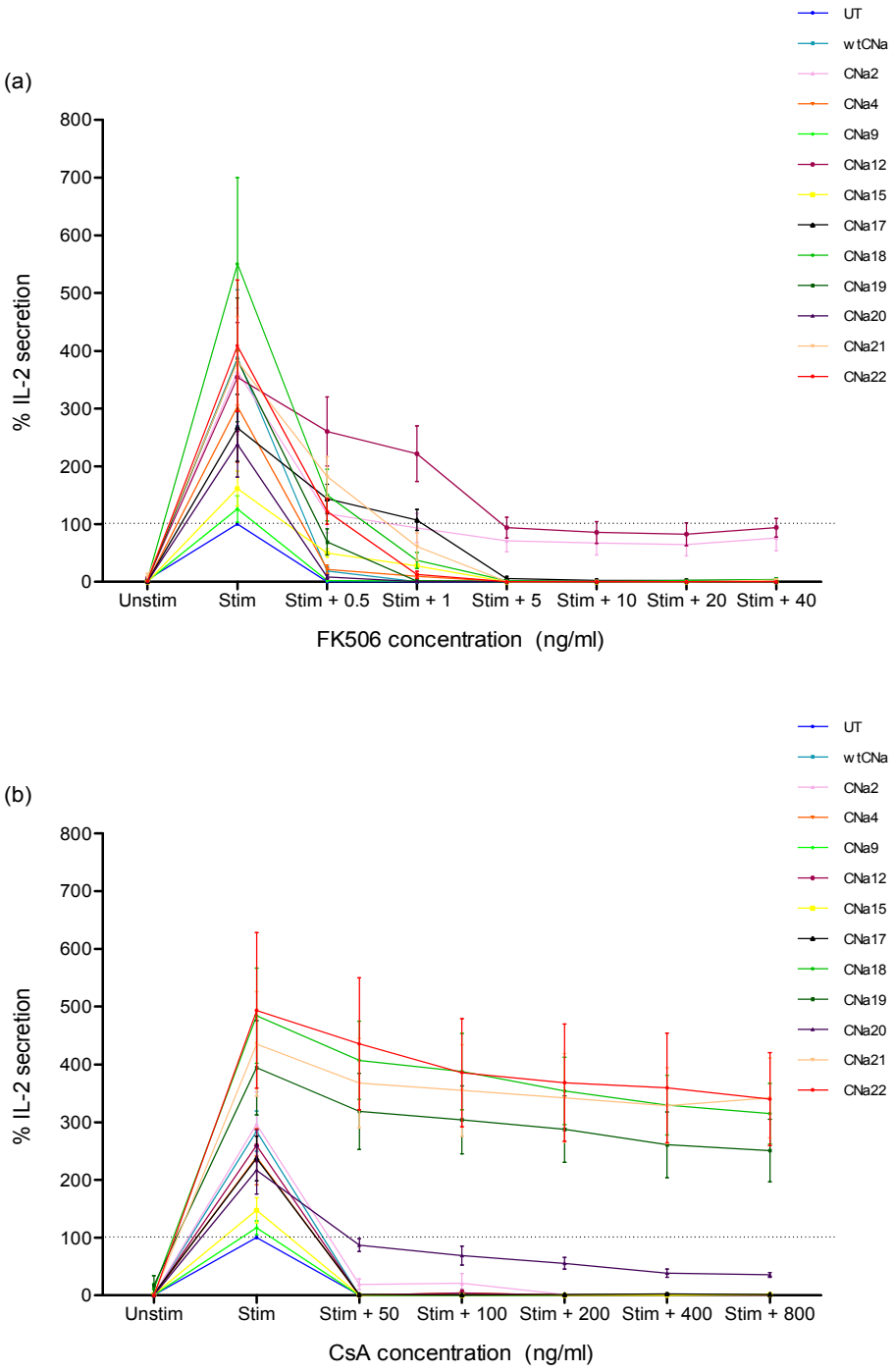


Figure 27: CNa mutants transduced, FACS sorted Jurkat cells are able to secrete IL-2 in the presence of FK506 (a) or CsA (b). Mean and SEM of three experiments shown. Dotted line indicates secretion by stimulated UT Jurkat cells without CN inhibitors (100%).

Cells transduced with CNb were also sorted to express equivalent GFP, and re-tested (see Figure 28). As measured by IL-2 secretion compared to UT without CN inhibitors, CNb30 conferred 85% resistance to 10ng/ml FK506 and 43% resistance to 200ng/ml CsA. It is striking that, despite sorting to a higher level of GFP expression in CNb transduced cultures, little over-expression of IL-2 was seen from CNb compared to CNa transduced cells. CNb26 and CNb30 resulted in some hyper-activity compared to stimulated untransduced cells without CN inhibitors (184/216% and 153/184% respectively in the FK506/CsA experiments), however this is much less than that seen from CNa transduced cells.

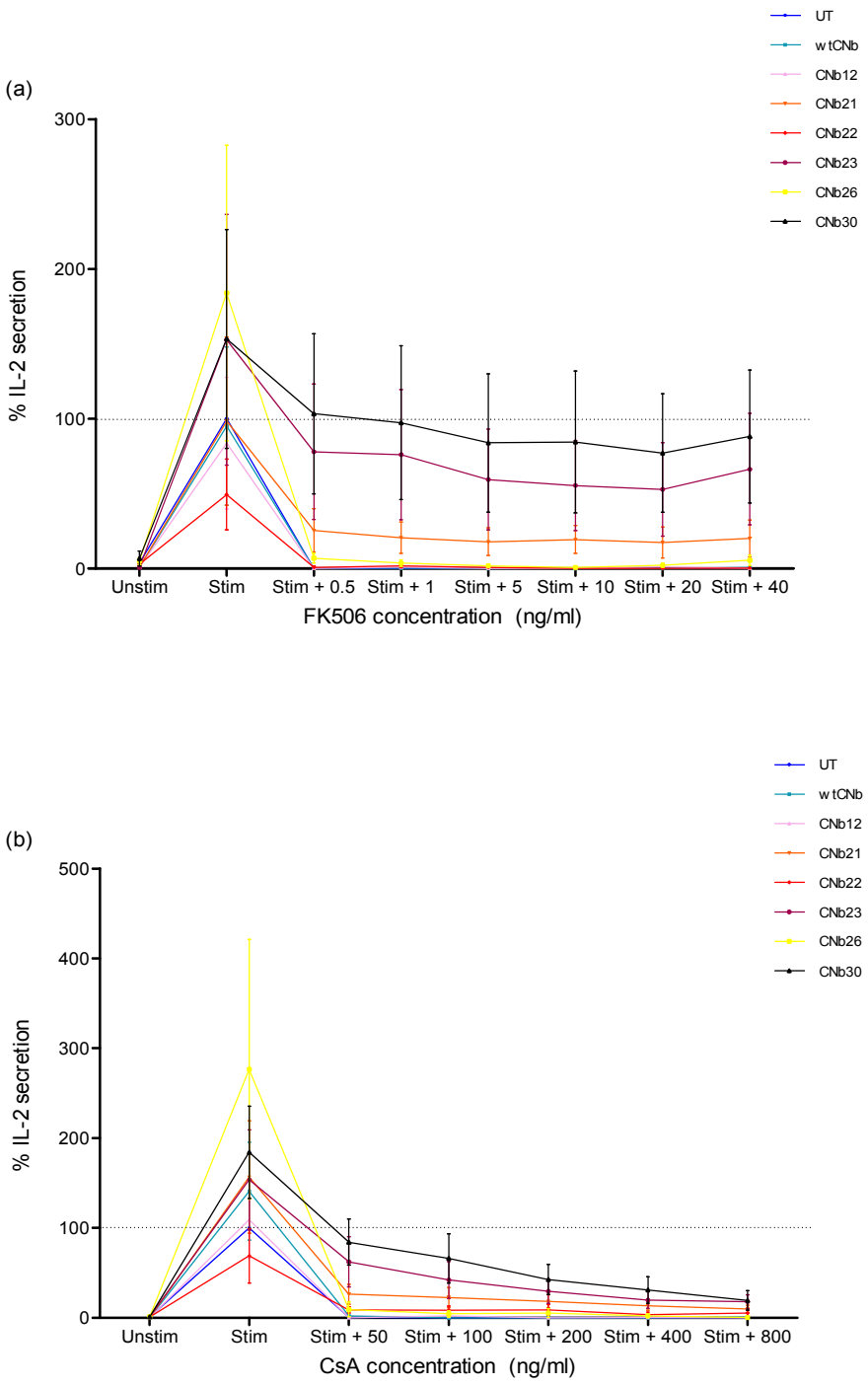


Figure 28: CNa mutants transduced, FACS sorted Jurkat cells are able to secrete IL-2 in the presence of FK506 (a) or CsA (b). Mean and SEM of three experiments shown. Dotted line indicates secretion by stimulated UT Jurkat cells without CN inhibitors (100%).

These data guided the selection of CNa12, CNa22 and CNb30 as those to pursue in further experiments. CNa12 conferred 85% resistance to FK506, CNa22 conferred 368% resistance to CsA and CNb30 conferred resistance to both CN inhibitors (85%/43% to FK506/CsA respectively).

3.3.7. Codon optimisation of selected CN mutants

To investigate the possibility of improving resistance by increasing expression of CN mutants, the selected constructs were codon optimised. This process altered the codon usage to reflect tRNAs that are commonly used in human cells, thus improving the efficiency of translation which may improve protein expression, potentially increasing resistance. In addition, during this process, GC content was raised to 70%, hairpins and literal repeats reduced to a minimum and splice sites avoided. An example of this codon optimisation is shown in Figure 29.

We also generated bi-cistronic constructs carrying both CNa and CNb mutants separated by a 2A sequence to investigate the effect of transduction with both genes together. Following generation of retroviral supernatant encoding codon optimised mutants, Jurkat cells were transduced, FACS sorted to equalise GFP expression, and CNa and CNb was quantified by Western blotting (see Figure 30).

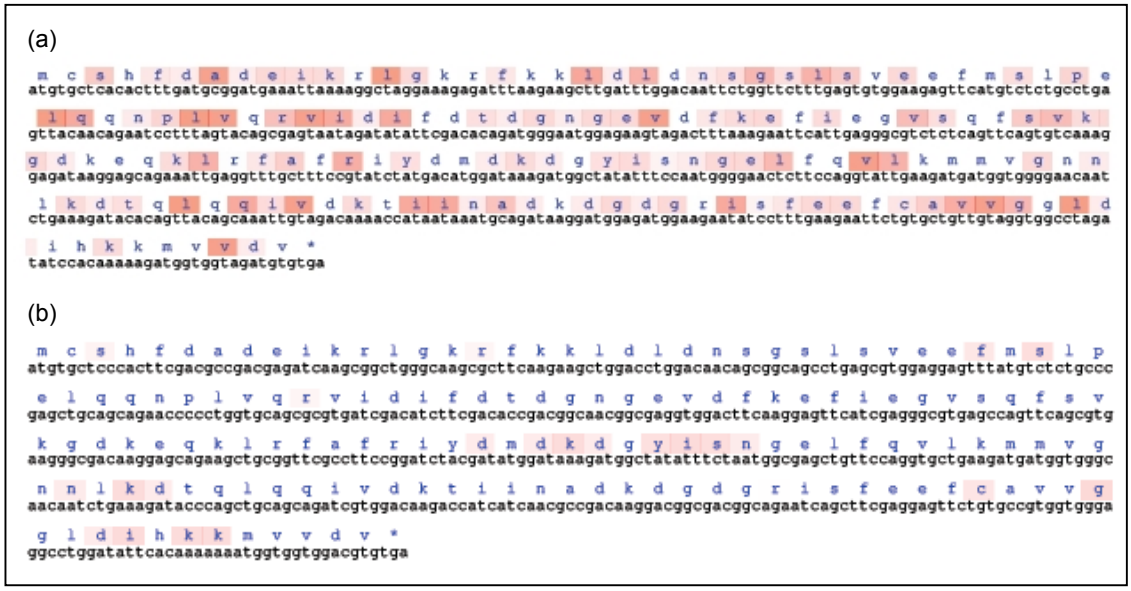


Figure 29: Example of codon optimisation of CN sequence. Panel (a) shows the original CNb sequence, panel (b) shows the optimised sequence. Amino acid sequence is shown on the top line, with the most common mammalian codons shaded in white, less common codons shown in pink and rare codons shown in red. The DNA sequence is shown below the coded amino acids.

Western blot data of Jurkats transduced with codon optimised CN mutants shows that transduction with CNa12 or 22 results in a 22-fold increase in expression of CNa, and transduction with CNb30 results in a 1.7 fold increase in CNb expression. Comparison with the increase in expression seen with non-codon optimised constructs (Table 7 and Table 8) suggests that codon optimisation increases the expression of CNa12 and CNa22 but has little effect on the expression of CNb30.

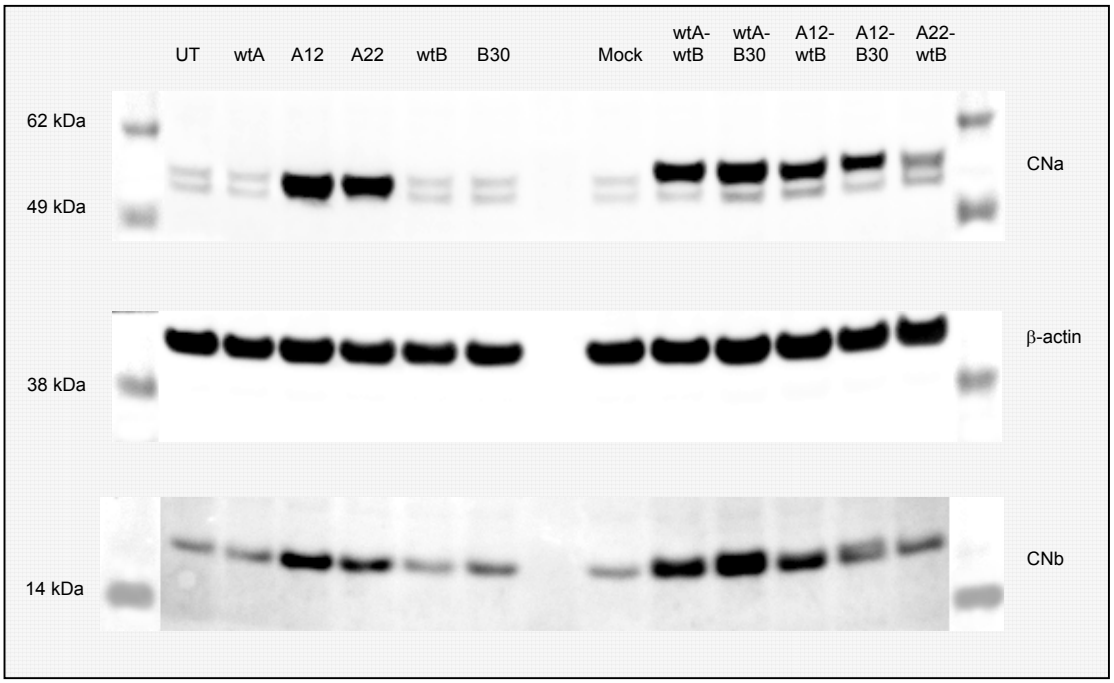


Figure 30: Jurkat cells transduced with codon optimised CN mutants express comparably high levels of CNa/CNb. Western blot performed following transduction and FACS sorting to the same level of GFP expression. β-actin is shown as protein loading control. Samples were stained for both CNa and CNb expression. Quantification of bands is shown in Table 9.

Interestingly, transduction of Jurkat cells with CNa mutants also resulted in a 3-4 fold increase in expression of CNb. In contrast, transduction of Jurkat cells with CNb did not result in increased expression of CNa; indeed CNb transduction resulted in a lower increase in CNb than that observed following CNa transduction. Transduction with bicistronic constructs containing both CNa and CNb led to increased expression of both proteins, however this increase was comparable to that seen after transduction with CNa alone (see Table 9).

Table 9: Expression of CNa and CNb in transduced Jurkats cells. Bands were quantified and normalised to β-actin expression to adjust for differences in protein loading. Final results are highlighted.

Sample	Actin	Relative actin	CNa	Relative CNa	CNb	Relative CNb	Adjusted CNa	Adjusted CNb
UT	942215	1.27	62776	49623	141396	111769	1.38	1.00
wtCNa	785263	1.05	65949	62550	188284	178581	1.74	1.60
CNa12	966436	1.30	1057383	814883	554607	427414	22.65	3.82
CNa22	800667	1.08	852371	792890	388578	361462	22.04	3.23
wCNb	744794	1.00	60214	60214	155441	155441	1.67	1.39
CNb30	828456	1.11	55634	50016	215322	193578	1.39	1.73
Mock	937877	1.26	45305	35978	154882	122996	1.00	1.10
wtCNa-wtCNb	1037667	1.39	712964	511736	511419	367075	14.22	3.28
wtCNa-CNb30	1091225	1.47	884004	603359	747623	510275	16.77	4.57
CNa12-wtCNb	1033276	1.39	508120	366257	412219	297131	10.18	2.66
CNa12-CNb30	813048	1.09	335321	307171	283882	260051	8.54	2.33
CNa22-wtCNb	803955	1.08	162674	150703	212742	197087	4.19	1.76

These data indicate that CNa is likely to be the limiting component of the CNa:CNb heterodimer, a theory postulated by Milan *et al* that is supported by the pattern of hyperactivity seen when transduced Jurkats are stimulated (Milan*, et al* 1994). Transduction of cells with additional CNa allows an overall increase in cellular CN, due to the presence of sufficient native CNb for heterodimer formation. Conversely, transduction with CNb does not lead to increased cellular CN heterodimer as limited CNa is available for binding, therefore introduced CNb must compete with native CNb. Increased overall CN may lead to a heightened response when the cell is stimulated and therefore increased IL-2 secretion. Figure 31 illustrates this hypothesis.

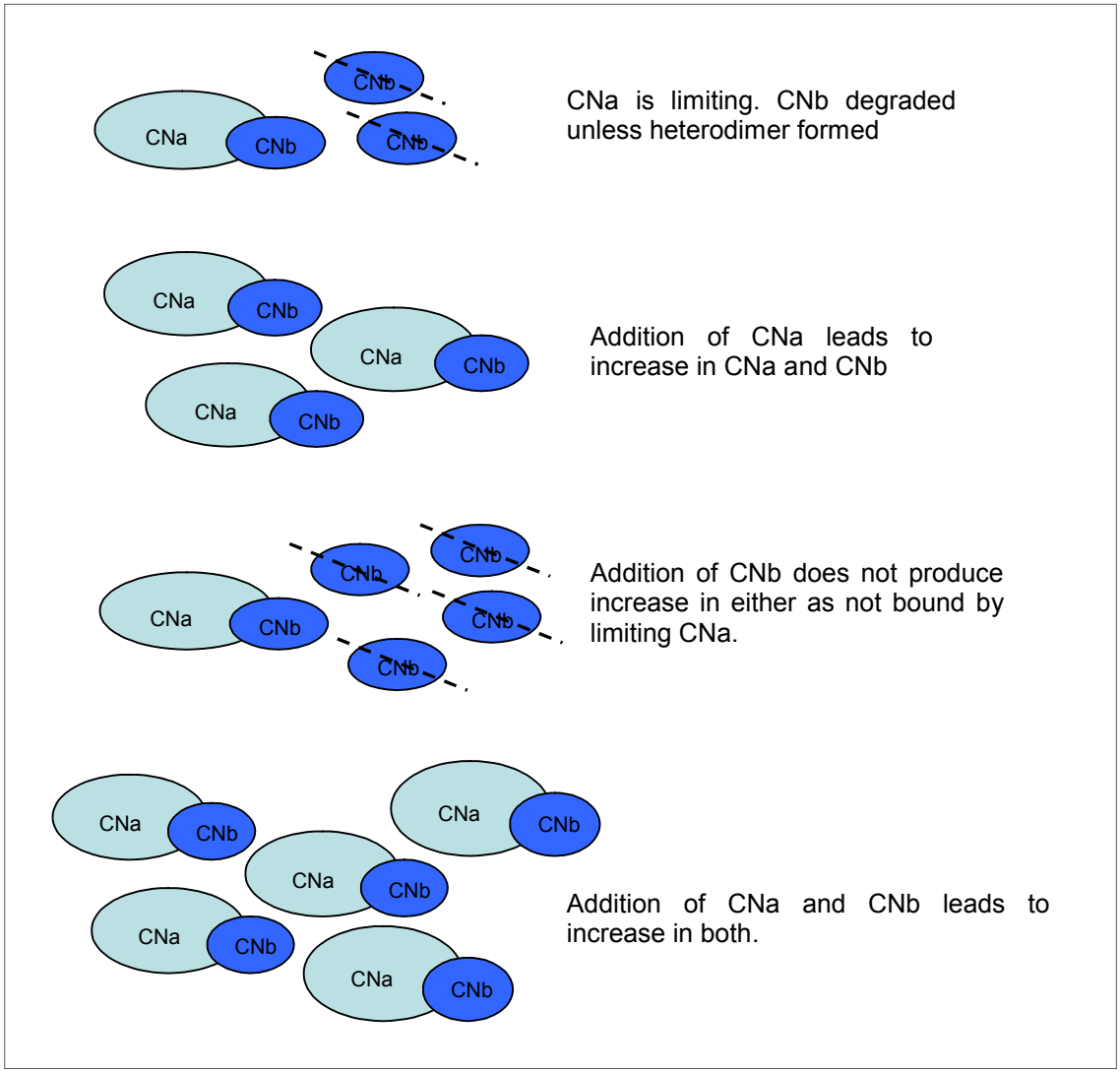


Figure 31: Illustration of CNa/b heterodimer formation. Relative excess of CNb compared to CNa results in CNa limiting the formation of CN heterodimer. Therefore transduction with CNa leads to increase in CNb, whereas transduction with CNb does not produce increase in either protein.

Following codon optimisation of selected CN mutants, resistance of Jurkat cells transduced with these mutants to CN inhibitors was re-tested. These results are shown in Figure 32 and summarised in Table 10. Jurkat cells transduced with codon optimised CN mutants showed a similar resistance profile to that seen with the corresponding non-optimised constructs. This is perhaps unsurprising for CNb30 as expression was equivalent between codon optimised and non-optimised constructs.

However, despite a considerable increase in the expression of CNa detected by Western blot, resistance to CN inhibitors was not increased after codon optimisation. It is possible that the limit of IL-2 secretion, and maximal resistance to CN inhibitors in this assay had been reached. A summary of the resistance profile observed in three experiments with non-optimised compared to codon optimised mutants is shown in Table 10. It is interesting to note that there is no further increase in hyper-activity as a result of either codon optimisation or co-expression of CNa and CNb together. As there is an increase in both CN expression and GFP expression (data not shown) following codon optimisation, we continued experiments with these sequences into primary cell lines.

Therefore, for testing in EBV-CTLs, CNa12 was selected to confer resistance to FK506, CNa22 for resistance to CsA, and CNb30 for resistance to both CN inhibitors. In addition the bicistronic construct of CNa12-CNb30 was selected which is able to render Jurkat cells resistant to both FK506 and CsA more effectively than CNb30 alone in the Jurkat IL-2 secretion assay.

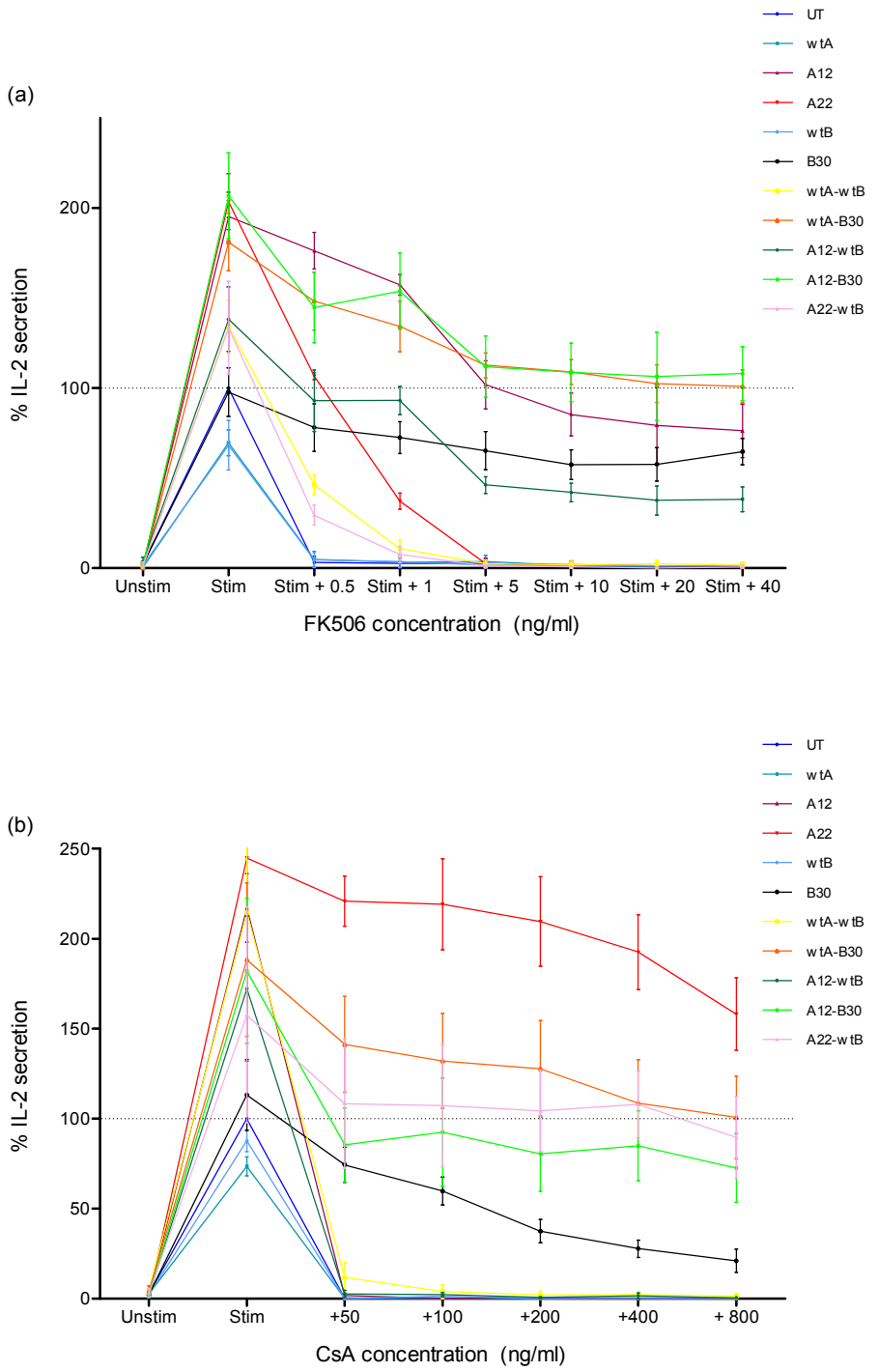


Figure 32: Transduction of Jurkat cells with codon optimised CN mutants allows IL-2 secretion in the presence of FK506 (a) or CsA (b). Mean and SEM shown *(n=3)*. Dotted line indicates secretion by stimulated UT Jurkat cells without CN inhibitors (100%).

Table 10: Summary of resistance conferred by codon optimised CN mutants. Jurkat cells retrovirally transduced with CN mutants were FACS sorted to equivalent MFI for GFP expression, stimulated with PMA and Ionomycin and IL-2 secretion in the presence of FK506/CsA was assessed by ELISA. IL-2 secretion was compared to that by stimulated untransduced cells in the absence of CN inhibitors to give % resistance. The resistance conferred by non-codon optimised and codon optimised constructs are shown. Data are the mean of three experiments.

CN mutant	% Resistance to FK506 pre optimisation	% Resistance to FK506 post optimisation	% Resistance to CsA pre optimisation	% Resistance to CsA post optimisation
UT	-	-	-	-
wtCNa	-	-	-	-
CNa12	85	85	-	-
CNa22	-	-	368	209
wCNb	-	-	-	-
CNb30	85	57	43	37
Mock	-	-	-	-
wtCNa-wtCNb	-	-	-	-
wtCNa-CNb30	-	108	-	127
CNa12-wtCNb	-	42	-	-
CNa12-CNb30	-	109	-	80
CNa22-wtCNb	-	-	-	104

3.4 Conclusions

In conclusion, we have designed and generated a panel of CN mutants to confer resistance to either or both of the CN inhibitors FK506 and CsA. Several rounds of mutant design were undertaken, combining those mutations that showed some effect in earlier rounds. We have established and optimised a transient luciferase screening assay in 293T cells to allow preliminary testing of all 54 CN mutants, which enabled selection of 17 mutants that confer resistance to FK506 or CsA.

Whilst a success rate of one third was achieved by this protein engineering with 17 of the 54 designed mutants retaining some activity in the presence of CN inhibitors, these activities did not always correlate to that predicted during design. For example, mutants CNa18-20 were intended to confer resistance to both CN inhibitors by

disruption of a residue involved in both interactions, however resistance to CsA only was observed. In contrast, mutants containing an insertion after CNb position 125 were anticipated to confer resistance to FK506 alone, however cells were also rendered resistant to CsA. These results demonstrate that the effect of amino acid mutations and insertions on the overall function of a protein can not easily be predicted, however successful design of resistant CN mutants was achieved.

Following identification of suitable mutants, we established and optimised a second screening assay to select those conferring the highest resistance to CN inhibitors in the Jurkat T cell line. Retroviral transduction of Jurkat cells with several of the CN mutants allowed IL-2 secretion in the presence of therapeutic doses of FK506/CsA, three of which were selected. These mutants were then codon optimised to improve expression. Despite this procedure, expression of CNb30 did not increase following codon optimisation. This is consistent with the hypothesis illustrated in Figure 31, demonstrating that CNa is required to stabilise CNb and that without increasing CNa, no additional CNb can be detected despite potential improvements to the efficacy of expression. In addition, no increase in resistance was noted following codon optimisation despite an increase in CNa12/22 expression. It is possible that the limit of this assay has been reached and that additional over-expression of CN can not further increase either IL-2 expression or resistance. For example, at lower levels of CN expression, our results indicate that CN is the limiting step in cellular activation when stimulated with mitogens. With increasing CN present in the cell, however, it is possible that CN reaches excess levels therefore the availability of Calmodulin, NFAT, or another component of the activation pathway may become limiting in cellular activation, thus preventing any effect of further increases in CN expression. Although we were unable to determine the effect of codon optimisation on resistance in this assay, we established that improvements were made in CNa expression following this process. In addition, screening in this Jurkat IL-2 secretion assay enabled selection of the three mutants that conferred the highest resistance to CN inhibitors for further study in EBV-CTL lines.

In Jurkat cells, transduction with wtCNa or CNa mutants resulted in increased IL-2 production by those cells upon stimulation with mitogens. It is likely that increased

CN expression leads to amplification of the activation signal, additional NFAT activity and a degree of hyper-reactivity to stimulation in this Jurkat cell line. Interestingly, Western blot data showed increased expression of CNb upon transduction with CNa and limited increase in CNb following transduction with CNb, indicating that CNa may be the limiting component of the CN heterodimer. This is supported by the observation that hyperactivity of Jurkat cells is observed to a higher degree upon CNa transduction compared to CNb transduction. However, it is important to note that no IL-2 secretion was observed in the absence of stimulation, indicating that these cells remain dependent on stimulation for activation, and that increased CN expression alone does not confer resistance, as demonstrated by transduction with wtCNa.

In summary, these results demonstrate that CNa and CNb mutants can be generated to confer resistance to either FK506, or CsA or both CN inhibitors. CNa12 conferred the highest levels of resistance to FK506, CNa22 to CsA and CNb30 conferred resistance to both drugs. Transduction with these mutants allows expression of Luciferase from 293T cells or IL-2 from Jurkat T cells in the presence of therapeutic doses of these drugs. These CN mutants were then tested in primary CTL lines.

3.5 Final conclusions

- We have successfully generated a panel of CNa and CNb mutants to confer resistance to FK506 or CsA in two cell line assays.
- Three mutants retaining the highest activity in the presence of therapeutic levels of FK506 or CsA were identified.
- Codon optimisation of these three mutants improved expression of CNa12 and CNa22 but not CNb30 in Jurkat cells, but did not improve the level of resistance observed to FK506 or CsA.
- Bicistronic vectors combining a CNa and CNb mutants may increase resistance further.

Chapter four

Evaluation of CN mutants in primary EBV-CTL lines

4.1 Aims

- To generate EBV-CTL lines from healthy donors.
- To transduce CTLs with CN mutants and evaluate the effect of transduction on phenotype, cytokine secretion and cytotoxicity.
- To assess the ability of the selected CN mutants to render EBV-CTL lines resistance to FK506/CsA.

4.2 Introduction

Following the identification of CN mutants capable of conferring resistance to CN inhibitors in Jurkat cells, the activity of these mutants in primary cells was examined. CN inhibitor resistant EBV-CTLs may be of therapeutic use in the treatment of PTLD post SOT, therefore this model was selected to examine resistance to CN inhibitors in primary cells. In addition, our laboratory has expertise in the generation and culture of EBV-CTLs, which can be maintained *in vitro* for a sufficient length of time to assay various end points.

In order to assess the effect of CN mutants, the effect of CN inhibitors on *in vitro* generated UT EBV-CTLs must be established. Whilst it is clear that pharmacological suppression of EBV immunity plays a role in the development of PTLD, previous studies have shown differential effects of CN inhibitors on *ex vivo* expanded T cells compared to naïve T cells when studying graft versus host disease (GVHD) (Contassot*, et al* 1998) and on committed CTLs compared to naïve CD8+ T cells in heart transplant rejection (Vaessen*, et al* 1994). These data suggest that activated cells are suppressed less effectively than naïve cells. In contrast, studies of CMV-CTLs derived from patients receiving immunosuppressive therapy have demonstrated that CMV specific CD8+ CTLs are present but display reduced functionality following

withdrawal from the patient, indicting that these cells are indeed suppressed by CN inhibitors (Engstrand*, et al* 2003). In addition, it has been shown that proliferation and secretion of IFN-γ by EBV-specific CTLs *in vitro* are affected by addition of CN inhibitors to culture conditions (Savoldo*, et al* 2001). Previous data regarding the effect of CN inhibitors on the cytotoxicity of CTLs is conflicting. Some studies find that, in contrast to proliferation and cytokine secretion, cytotoxicity is unaffected by the presence of FK506 or CsA (Savoldo*, et al* 2001), whereas others have shown that cytotoxicity is inhibited by long term culture in CN inhibitors (Zhan*, et al* 2003). In summary, data on the effect of CN inhibitors on the functionality of *ex vivo* expanded EBV-CTLs are conflicting, particularly with regard to cytotoxicity. Further work is thus required to clarify the effects of CN inhibitors on EBV-CTLs. We have therefore initially studied the effect of CN inhibitors on untransduced EBV-CTL lines expanded *in vitro* from healthy donors in order to identify end points that are affected by these drugs to allow determination of whether transduction with CN mutants restores these suppressed functions.

The potential effects of transgene expression within the target cells have also been evaluated. Calcineurin is critically involved in the activation of T cells, therefore several possibilities could arise from increased expression of this gene. Higher levels of CN may lead to increased NFAT activity and hyper-responsiveness to stimulation, as shown in Jurkat cell lines. Alternatively, if Calmodulin or NFAT are saturated, additional CN expression may have no effect on T cell activation. As demonstrated in chapter three, transduction with CNb mutants appears to lead to only a limited increase in intracellular CN levels, and no hyperactivation in Jurkat cells, therefore mutants of this gene may be less likely to confer side effects of increased CN activity. We have additionally addressed the important issue of whether transduction with CN mutants confers on EBV-CTLs the ability to proliferate independently of specific antigen stimulation. As cellular activation requires the interaction of several independent pathways, we felt that increased signalling through CN was unlikely to render EBV-CTLs stimulation independent and lead to inappropriate growth, and have demonstrated this to be the case. Finally, as primary EBV-CTLs are more biologically relevant than transformed cell lines and therefore subject to normal cellular regulation mechanisms, hyperactivation of CN may lead to the induction of activation induced

cell death or anergy, rather than increased proliferation. Although cell lines are a useful model for initial *in vitro* studies, the effect that these manipulations may have on primary cells can not always be accurately predicted. Therefore we examined the effect of transduction with CN mutants on EBV-CTLs in both the presence and absence of CN inhibitors.

4.3 Generation of EBV-CTL lines

4.3.1 Generation and characterisation of EBV-CTLs

Several optimisation experiments were performed to establish the EBV-CTL model and determine suitable end points for identifying resistance to CN inhibitors. First we confirmed that generation of EBV-specific CTLs was possible from healthy donors, and that these cells could proliferate, secrete cytokines and kill autologous LCL targets. Next the effect of FK506 on proliferation and cytokine secretion was determined to identify measures of suppression by and resistance to CN inhibitors.

EBV CTL lines were generated from healthy donors as outlined in methods. In response to weekly stimulation with irradiated autologous LCL, good proliferation of EBV-CTLs was observed as shown for two representative donors in Figure 33, where expansion from 10^6 to $32/53x10^6$ CTLs occurred over 5 weeks. As noted by other investigators (Wilkie*, et al* 2004), the proliferation rate of EBV-CTL lines is variable between donors with typical expansion of approximately 2-3 fold per week. Therefore, sufficient cells to perform functional assays can consistently be generated following 4-5 weeks culture.

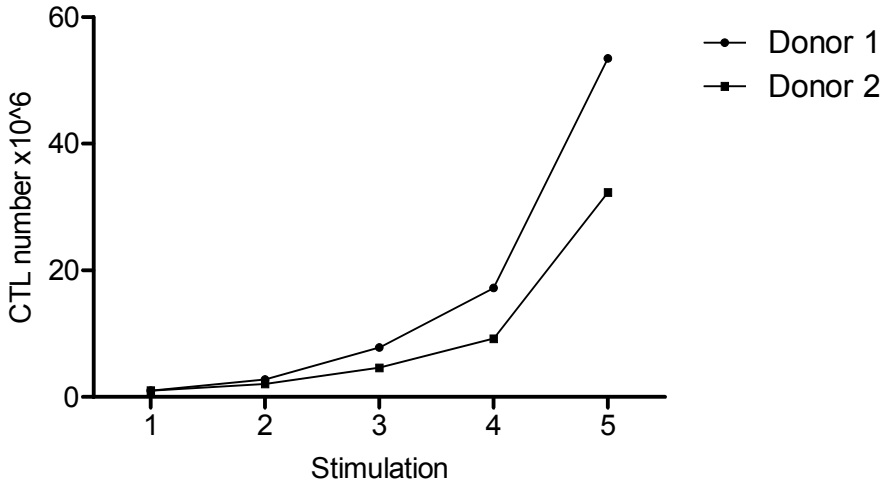


Figure 33: Expansion of EBV-CTLs *in vitro*. EBV-CTL proliferation in response to weekly stimulation with autologous LCL was measured for 5 weeks. Two representative donors are shown.

Supernatant from EBV-CTL cultures harvested 24 hours post stimulation was examined to assess secretion of both IL-2 and IFN-γ. We established that these EBV-CTL lines secreted low or undetectable levels of IL-2, but high levels of IFN-γ that was easily measurable by both ELISA (data not shown) and ELISPOT (see Figure 34), with over 9000 spot forming cells per 10^5 identified in two CTL lines tested. This IFN-γ secretion was shown to be secreted specifically in response to stimulation with autologous LCL and not in response to stimulation with MHC-mismatched third party (allogeneic) LCL, demonstrating that CTLs generated using this protocol are MHC-restricted.

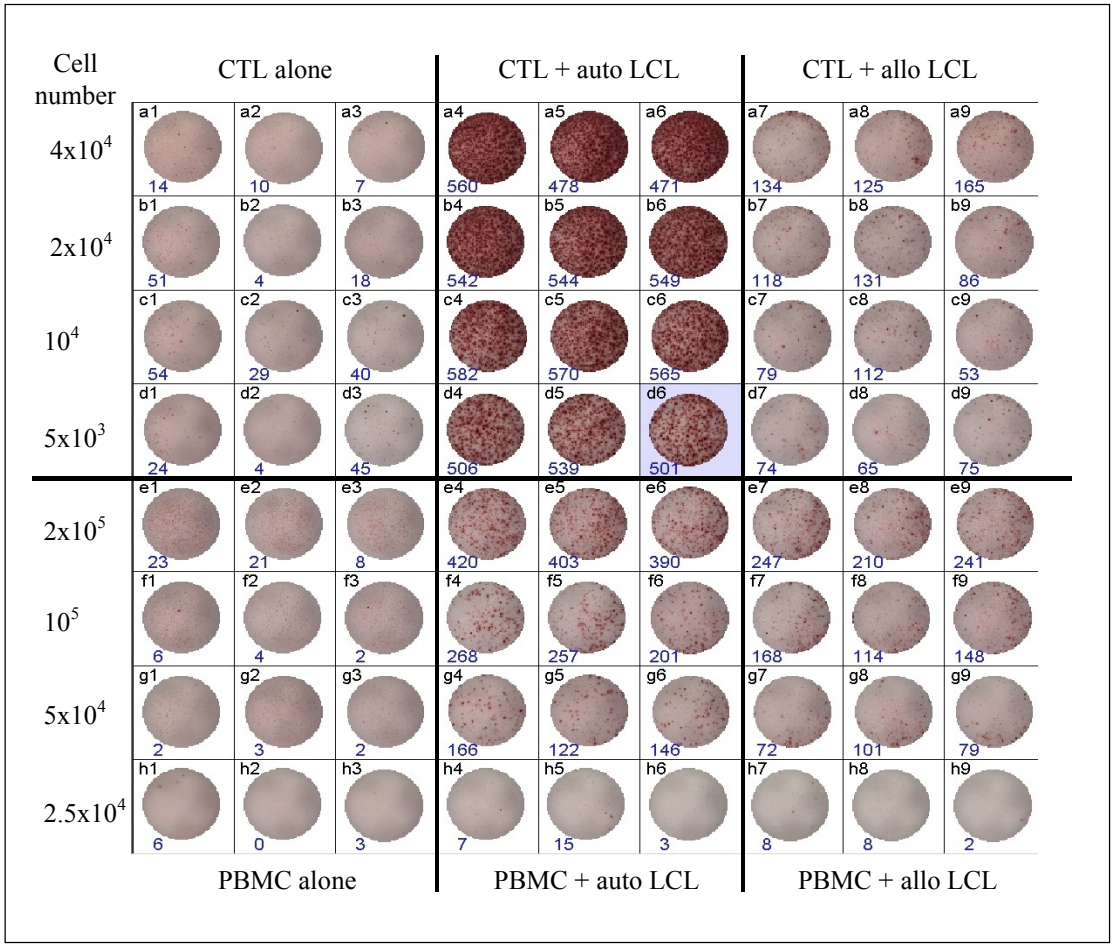


Figure 34: IFN-γ secretion from EBV-CTL line. (a) IFN-γ secretion in response to stimulation with autologous or allogeneic LCL was measured by ELISPOT. Wells tested in triplicate. 4 doubling dilutions of CTLs were tested from $4x10^4$ to $5x10^3$ per well, 3 doubling dilutions of PBMC tested from $2x10^5$ to $5x10^4$ per well both alone, or with either autologous/allogeneic LCL stimulators. One representative donor of three is shown.

Finally, cytotoxicity assays were performed to confirm that killing by these EBV-CTL lines was EBV-specific and MHC restricted, examining cytotoxicity towards either autologous or allogeneic LCL targets. HSB-2 target cells were included to determine the level of LAK/NK-mediated killing by these lines. Figure 35 shows that EBV-CTL lines are indeed MHC restricted, demonstrating high killing of autologous targets, with a range of between 40 and 60% of maximal release at effector:target ratios of 30:1. Killing of allogeneic LCL was low (consistently <10%) showing that the observed cytotoxicity was MHC restricted. The degree of HSB-2 lysis seen was generally low

but variable between donors, ranging from 5-45%, reflecting the low level of LAK/NK activity in the majority of CTL lines. Following establishment of a reliable protocol for expansion of EBV-specific T cells to generate EBV lines from healthy donors, the effect of introducing FK506 into culture conditions was examined.

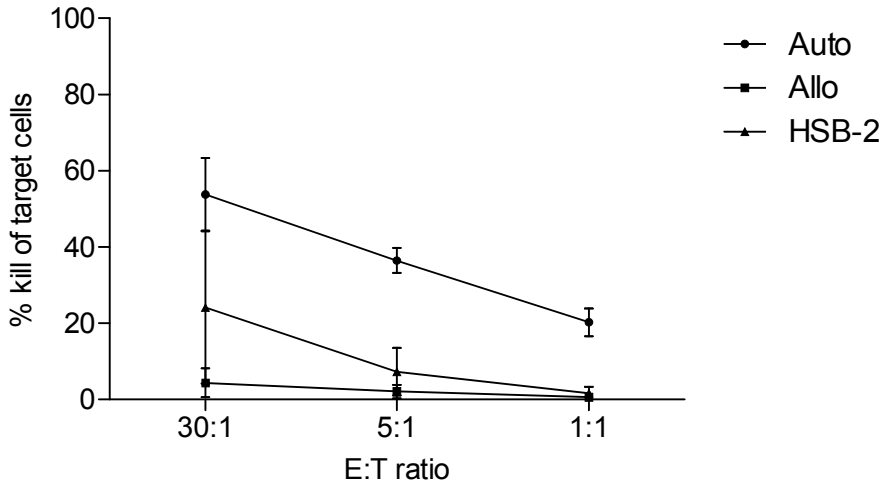


Figure 35: EBV-specific cytotoxicity of EBV-CTL lines. Killing of autologous or allogeneic LCL targets, or HSB-2 cells was measured by ^{51}Chromium release in a standard 4 hour assay. Mean and SEM shown (*n=3*). E:T = effector to target cell ratio.

4.3.2 Suppression of untransduced EBV-CTLs by calcineurin inhibitors

To confirm that EBV-CTL lines were suppressed by the addition of CN inhibitors, increasing concentrations of FK506 were added to culture conditions and the effect on proliferation and cytokine secretion was assessed. IFN-γ was chosen as a candidate end point for measurement to monitor suppression by CN inhibitors, due to both the high level of secretion observed from EBV-CTLs following stimulation, and the presence of NFAT responsive elements in the promoter region of the IFN-γ gene (Kiani*, et al* 2001). As NFAT is involved in regulation of IFN-γ expression, it was anticipated that addition of either CN inhibitor to UT EBV-CTL culture conditions would result in reduced IFN-γ secretion. Secretion of IFN-γ was assayed by ELISA 24 hours after EBV-CTL stimulation in the presence of 0, 1 or 10ng/ml FK506. This

confirmed a dose dependent reduction in the level of IFN-γ secretion. When compared to secretion in the absence of FK506, a mean 24% secretion with 1ng/ml and 10% secretion with 10ng/ml was observed (see Figure 36).

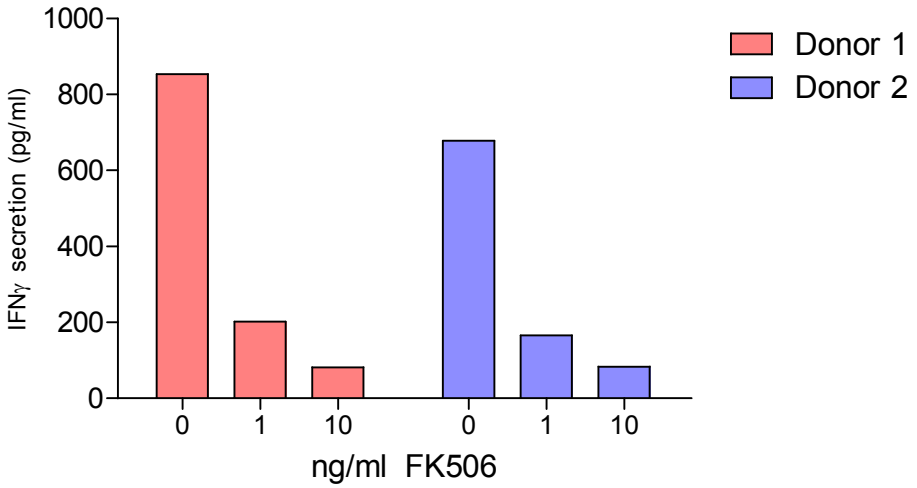


Figure 36: IFN-γ secretion by EBV-CTLs is prevented by addition of FK506. Two EBV-CTL lines were stimulated with autologous LCL in the presence of 0, 1 or 10ng/ml FK506. Secretion of IFN-γ was assessed after 24 hours by ELISA.

To assess the effect of CN inhibitors on EBV-CTL expansion, increasing doses of FK506 were added to two EBV-CTL lines at third stimulation, and growth was monitored for three further weeks. FK506 concentration was measured and found to have a half life of approximately 3 days in CTL cultures (data not shown). Therefore, FK506 was changed half weekly synchronous with half media and IL-2 change. As shown for IFN-γ production, a dose dependent reduction in proliferation was observed. Compared to growth in the absence of FK506, EBV-CTL expansion was reduced to 66% in 0.1ng/ml, 20% in 1ng/ml, 7% in 5ng/ml and <5% in 10 or 20ng/ml FK506 (see Figure 37).

These experiments confirm that EBV-CTL lines generated from healthy donors are indeed susceptible to suppression by FK506 *in vitro*, which is measurable by monitoring both cellular proliferation and secretion of IFN-γ. Therefore these end points were used to detect resistance to CN inhibitors conferred by transduction with CN mutants.

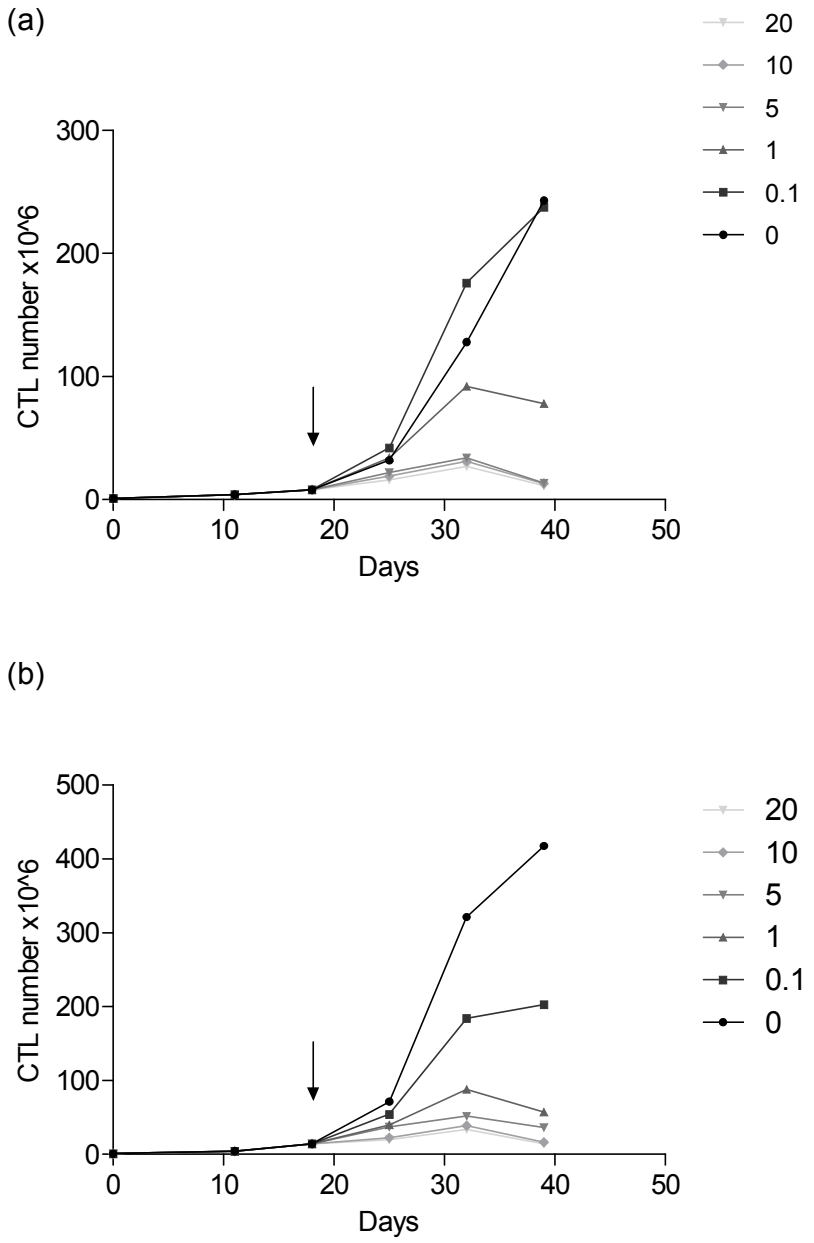


Figure 37: Proliferation of EBV-CTLs during *in vitro* culture is inhibited by FK506. Two EBV-CTL lines were established and increasing concentrations of FK506 were added from stimulation 3 onwards (indicated by arrow). Cells were counted and re-stimulated weekly. FK506 was changed half weekly. Proliferation of two representative donors is shown (panels a and b).

4.4 Transduction of EBV-CTL lines

4.4.1 Transduction of EBV-CTL lines with retroviral vectors

Following optimisation of generation of untransduced EBV-CTLs and demonstration of suppression by CN inhibitors, the optimal conditions for CTL transduction were determined. Based on published data and previous experience in our laboratory, CTLs were transduced with retroviral supernatants using Retronectin (RN) and spinfection as outlined in methods. This was performed 3-4 days after the second or third stimulation with LCL, following the same protocol used for Jurkat transduction. This time point was chosen because it allows establishment of EBV-specificity of CTL lines but leaves sufficient time to assess expansion of transduced CTLs over the subsequent five weeks without exhaustion of the line (Wilkie*, et al* 2004). Several variables were investigated to maximise transduction efficiency, including virus loading onto RN prior to addition of target cells, additional centrifugation on days subsequent to transduction, and performing two rounds of transduction on consecutive weeks.

Previous work by Quintas-Cardama investigating the optimal procedure and conditions for RV transduction of T cells led to the following conclusions: pre-loading of RV onto RN prior to addition of T cells does not increase transduction efficiency, additional spinoculation on two consecutive days following transduction improves transduction efficiency and spinoculation at room temperature or higher is most effective (Quintas-Cardama*, et al* 2007). We therefore examined the effect of these alterations to our transduction protocol in two EBV-CTL lines. Our data are in agreement with Quintas-Cardama regarding pre-loading of RV onto RN, with no benefit of this additional step observed (Table 11a). In contrast, we observed no increased transduction efficiency when further spinoculations were performed following the initial procedure (Table 11b). There are several differences between our investigations and the study of Quintas-Cardama that could account for this discrepancy. Firstly, their study investigates gene transfer into T cells that are non-specifically activated with PMA or CD3/CD28 beads, which results in activation of all T cells present, whereas we have generated and transduced EBV-specific cells only. It

is likely that a main factor limiting transduction efficiency into antigen specific T cells is the proportion of cells that are dividing during the period that infectious virus is present. In addition, Quintas-Cardama utilise RV pseudotyped with the GALV envelope protein rather than RD114. GALV pseudotyped virus has a 13hr $T_{1/2}$ (Uckert, *et al* 2000) therefore additional manipulations 24hr post transduction when little viable virus is remaining would not be expected improve transduction efficiency. In contrast, RD114 pseudotyped retroviruses are more stable at 37^{o} with a $T_{1/2}$ of over 24hr (Uckert, *et al* 2000), therefore additional spinoculations might be anticipated to be more effective. However, we found that additional spinoculation with RD114 pseudotyped virus did not improve transduction efficiency using our protocol. Therefore we continued performing transduction with no pre-loading of RN, and no additional centrifugation steps.

Table 11: Optimisation of CTL transduction in two cell lines. (a) Effect of pre-coating RN with SFG_GFP retrovirus before addition of CTLs and additional RV. (b) Effect of additional spinfection. Transduction efficiencies as assessed by flow cytometry for GFP performed 7 days after transduction from two CTL lines are shown.

(a)

Sample	Not pre-loaded	RN pre-loaded
Donor 1	27%	26%
Donor 2	34%	34%

(b)

Sample	Spun once	Spun twice
Donor 1	58%	60%
Donor 2	62%	63%

Following optimisation of the transduction protocol, we assessed the effect of performing an additional round of transduction one week after initial transduction. EBV-CTLs were transduced with a variety of retroviral vectors either once only following the second stimulation, or following both the second and third LCL stimulations. GFP expression increases for the first week after transduction and then stabilises. Transduction efficiency resulting from a single round of infection was 50-65% (see Figure 38a). Performing a second round of transduction improved this to 70-

85% (see Figure 38b), therefore as measured by the proportion of cells that are expressing the transgene a 20% increase was observed as a result of performing two rounds of transduction.

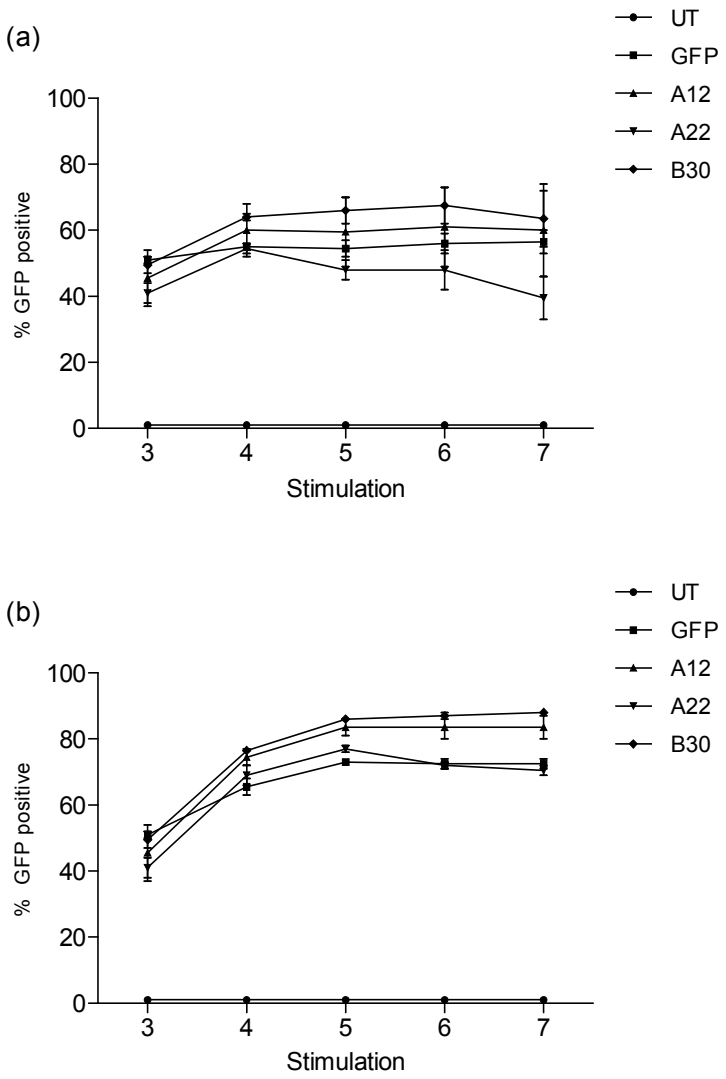


Figure 38: Increase in transduction efficiency with two compared to one round of transduction. Two EBV-CTL lines were transduced with retroviral vectors encoding GFP alone or with CN mutants A12, A22 or B30. GFP expression was measured by flow cytometry at each stimulation following either one (a) or two rounds (b) of transduction. Mean and SEM shown (*n=2*).

However, it was subsequently noted that CTLs undergoing two rounds of transduction proliferated considerably slower than those transduced once. Over three weeks following a single round of transduction, two CTL lines expanded between 128 and 255 fold, whereas in the three weeks following two rounds of transduction the same CTL lines expanded 40-70 fold (see Figure 39a and b). There are two likely explanations for this observation: it is possible either that expression of a higher level of transgene is detrimental to the cells and could inhibit proliferation/lead to cell death, or that the additional manipulation involved in the transduction procedure itself has a negative effect on the cells. The former suggestion would also be expected to lead to a reduction in the overall proportion of transduced cells with time, as they would have a proliferative disadvantage compared to the untransduced cells within the same line. As this is not observed (Figure 38b), the possibility that CTLs are sensitive to increased manipulation *in vitro* may be more likely. This is confirmed by the observation that UT cells also proliferate more slowly after two rounds of mock transduction. Therefore, since good transduction efficiency can be achieved by only one infection, in further experiments EBV-CTLs were transduced once following the second stimulation, GFP expression was measured after 7 days and subsequent experiments and monitoring were performed from the fourth stimulation onwards.

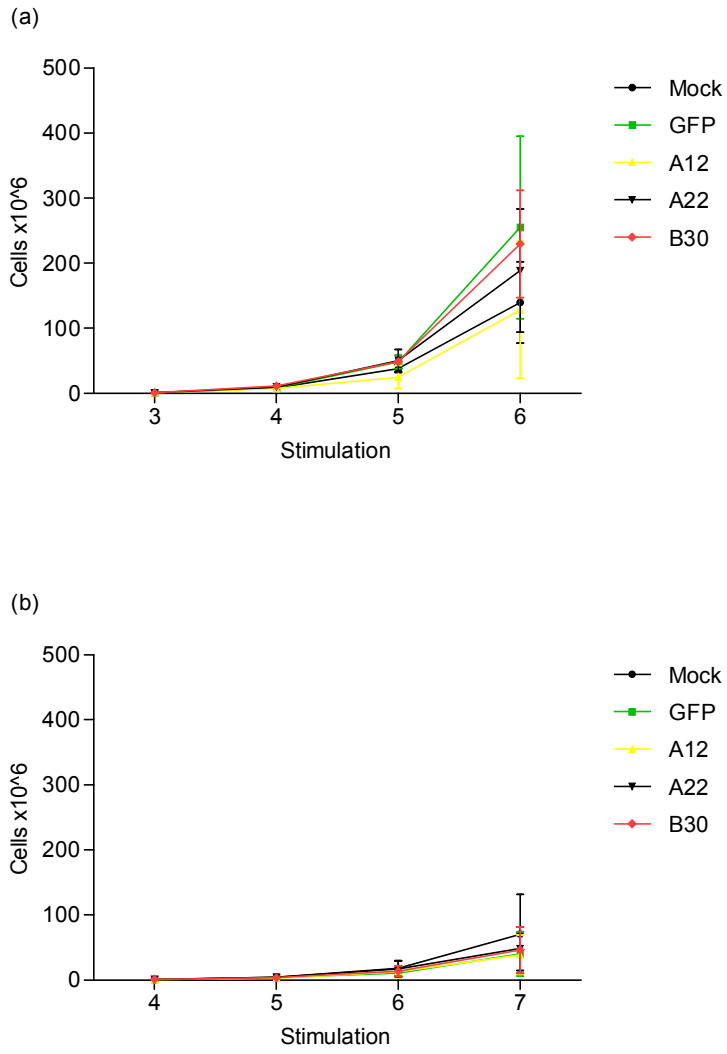


Figure 39: EBV-CTL proliferation is reduced following two rounds of transduction. Cell growth was monitored for three weeks following either one (a) or two (b) rounds of transduction with retroviral vectors encoding GFP alone or with the CN mutants shown. Mean and SEM shown (*n=2*).

4.4.2 Generation of stable retrovirus producer lines

Following optimisation of EBV-CTL generation and transduction, cell lines stably producing RV encoding the selected CN mutants were generated. This allows the production of large volumes of consistently high titre RV supernatant. As illustrated in Figure 40, multiple transductions of the packaging cell line FLYRD18 were performed

with GALV pseudotyped CN retrovirus. This resulted in a bulk population of FLYRD18 cells, likely with several RV integrants per cell, expressing high levels of CN and GFP. These cells were FACS sorted to obtain those with the highest MFI and clonal populations were produced by limiting dilution (this procedure was performed by Dr Martin Pule). These clones were assayed flow cytometrically for GFP expression and those displaying the highest MFI were selected. Titration of supernatants from these highly transduced cells allowed identification of those that produced the highest titre RV. These clones were selected for use in subsequent experiments.

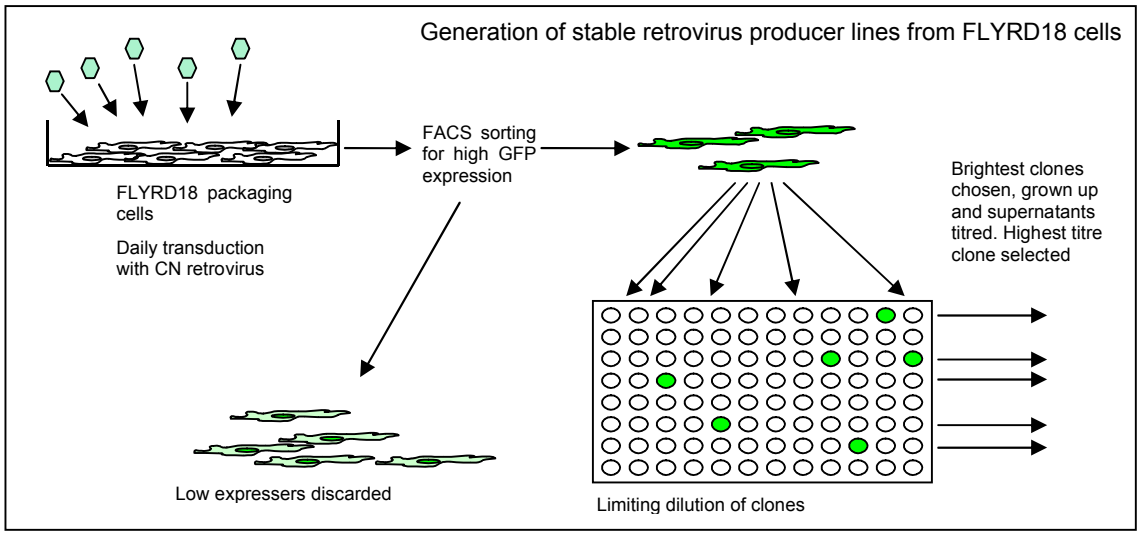


Figure 40: Schematic illustration of high titre stable retrovirus producer line generation. Multiple transductions of FLY-RD18 packaging cells were performed, followed by limiting dilution and selection of the highest GFP expressing cells.

Following generation of stable producer lines, supernatant was titred on 293T cells alongside transiently produced RV supernatant for comparison. As shown in Figure 41, supernatant from stable producer cell lines contained half to one log more infectious particles per millilitre compared to virus generated using a standard transient method of production. Using a standard transduction procedure of 2ml RV supernatant per $0.5x10^6$ CTLs, supernatants with a titre of $\sim 6x10^5$ as produced by these stable producer lines result in a multiplicity of infection of 2.5 infectious

particles per cell, compared with approximately 0.6 achieved using transient retroviral supernatant.

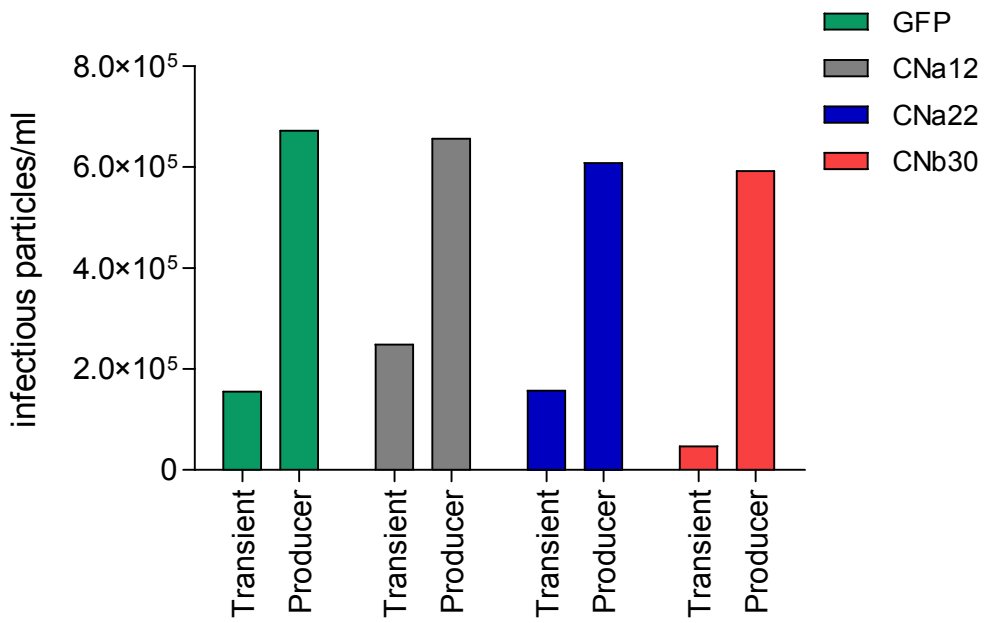


Figure 41: Stable producer lines generate higher titre RV supernatant compared to transient methods. Transient retroviral supernatants and those produced by stable retroviral producer lines were titred in parallel on 293T cells. Half to one log increase is seen from stable producer supernatants.

Following these optimisation experiments, the protocol for generation of EBV-CTLs, the end points for determining sensitivity to CN inhibitors and the procedure for transduction of EBV-CTLs were established. In addition, stable RV producing cell lines had been generated. Subsequently, the effect of transduction with CN mutants CNa12, CNa22 and CNb30 in primary EBV-CTL lines from healthy donors was investigated.

4.5 Evaluation of CN mutants in primary EBV-CTL lines

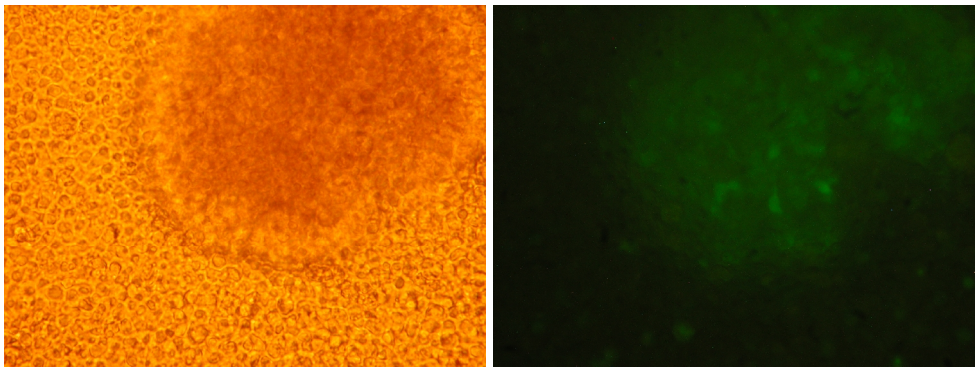
4.5.1 Transduction of primary EBV-CTL lines with CN mutants

EBV-CTL lines were generated from five healthy donors and transduced after two stimulations with RV supernatant produced from FLYRD18 stable producer lines encoding GFP alone or with CNa12, CNa22 or CNb30 transgenes. Transduction efficiency was assessed by flow cytometry to determine GFP expression 7 days following infection. Transduction efficiencies of between 52 and 82% were seen for all 5 donors, with a similar proportion of transduced cells for all constructs, but an increased MFI observed with the SFG_GFP alone vector compared to vectors also containing CN mutants (see Table 12 and Figure 42). This is likely to be due to GFP expression being driven by viral LTRs in the control construct, but following a transgene utilising an IRES in the mCN test constructs, as translation from an IRES is less efficient than that driven directly by the LTR.

Table 12: Comparable transduction efficiency of five EBV-CTL lines transduced with CN mutants. GFP expression in CTL lines was assessed by flow cytometry 7 days after transduction. Percent transduced cells is shown.

Donor	GFP alone	CNa12	CNa22	CNb30
1	54	52	56	63
2	56	68	53	65
3	77	75	81	82
4	63	66	64	73
5	68	60	60	74
Mean	64%	64%	63%	71%

(a)



(b)

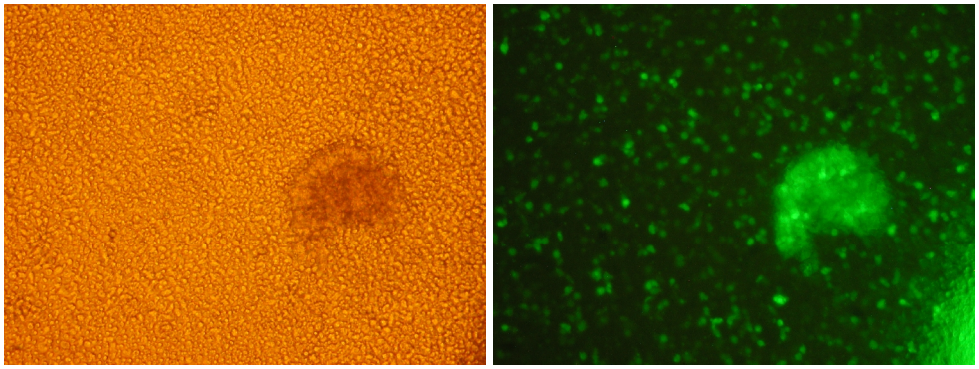


Figure 42: Light and UV microscope images of transduced EBV-CTLs. (a) Transduced with SFG_CNb30_eGFP and (b) SFG_eGFP alone control. 20x magnification.

Following improved resistance in Jurkat cells when CNa and CNb mutants were expressed together, EBV-CTLs were also transduced with the bicistronic construct CNa12-CNb30. However, reduced transduction efficiency was observed when CTLs were transduced with both genes compared to transduction with CNa or CNb alone. It is possible that this was due either to production of retroviral supernatant with a lower titre, or to lower expression of the eGFP reporter from the triple construct following two transgenes. The contribution of these factors is difficult to separate however, as expression of eGFP is the parameter used to titre the retroviral preparations. Furthermore, cells transduced with the triple CNa_CNb_eGFP construct proliferated poorly following transduction (data not shown). It is possible that expression of both CN genes in primary EBV-CTLs leads to greatly increased CN expression, therefore

stimulation with autologous LCL results in amplified signalling to the nucleus leading to activation induced cell death. Due to these poor initial results with bicistronic constructs, transduction with both CN genes concurrently was not explored further.

4.5.2 Transduction with CN mutants does not affect proliferation or phenotype of CTL lines

To determine the effect of transduction with CN mutants on the characteristics of the cell population, the phenotype of the EBV-CTL lines was examined. Expression of cell surface markers was assessed by flow cytometry four days after stimulation 5. Expression of CD3/4, CD3/8, CD16/56, CD3/16/56 and CD25 were unchanged between transduced and untransduced lines (see Figure 43a, $p=0.77$, two-way ANOVA), indicating that transduction and expression of the CNb30 transgene does not alter the CD4/CD8 subset distribution, NK/CIK content or activation status of EBV-CTL lines. In addition, the distribution of memory subsets was analysed and no difference was observed in the proportion of central memory, effector memory, naïve or terminally differentiated cells between transduced and untransduced CTLs (see Figure 43b, $p=0.984$, two-way ANOVA). These results confirm that the phenotype of these EBV-CTL lines was predominantly CD8+, central or effector memory T cells, and importantly that this was not affected by transduction with CN mutants.

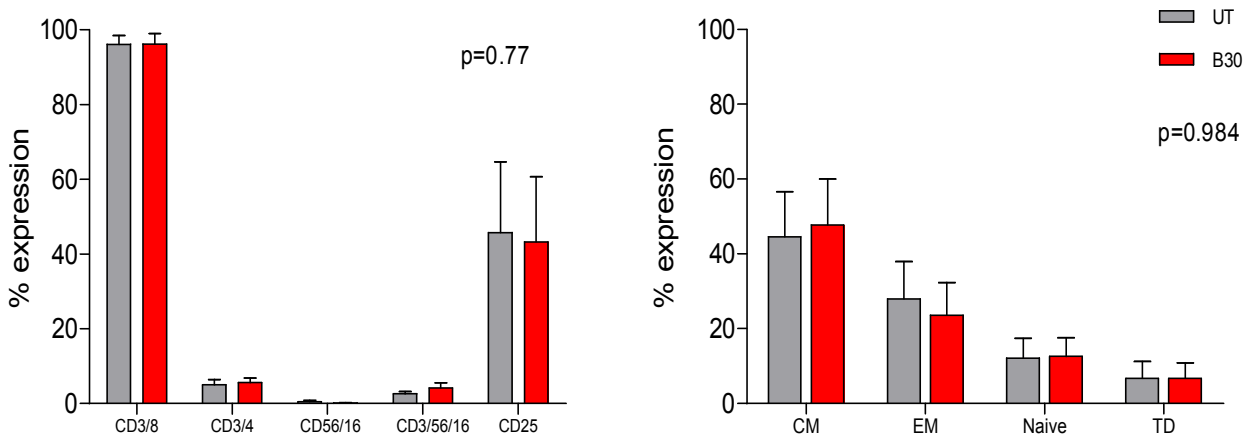


Figure 43: Expression of phenotypic markers on EBV-CTLs is not affected by transduction with CNb30. (a) Four days following their 5th stimulation, CTLs were analysed for surface expression of CD3, 4, 8, 16/56 and CD25 by flow cytometry. (b) Distribution of memory subsets in UT and CNb30 transduced CTL lines. CM=Central memory (CD45RO+, CD62L+), EM=Effector memory (CD45RO+, CD62L-), Naïve (CD45RO-, CD62L+), TD=Terminally differentiated (CD45RO-, CD62L-). Mean + SEM of 5 CTL lines shown.

EBV-CTL lines transduced with CN mutants were cultured in the absence of immunosuppression to examine the effect of transduction on cell growth. Transduction was performed three days after the second stimulation, transduction efficiency was assessed one week later and cell growth monitored from stimulation four for three weeks. EBV-CTL lines proliferate well *in vitro* for approximately 7-8 stimulations, at which point senescence generally occurs and a plateau is observed (Wilkie*, et al* 2004). Therefore, *in vitro* experiments were terminated at stimulation 7 to avoid this plateau confounding the results obtained. No significant difference was observed between UT CTL expansion and those transduced with either GFP alone or any of the three CN mutants (see Figure 44). 85 fold expansion was observed for UT CTLs, and between 66 and 100 fold expansion for the transduced CTLs (*p=0.96*, expansion of all CTL lines compared with one-way ANOVA). These results demonstrate that no proliferative advantage is conferred upon EBV-CTLs by transduction with CN mutants, or any disadvantage as a result of a single transduction procedure.

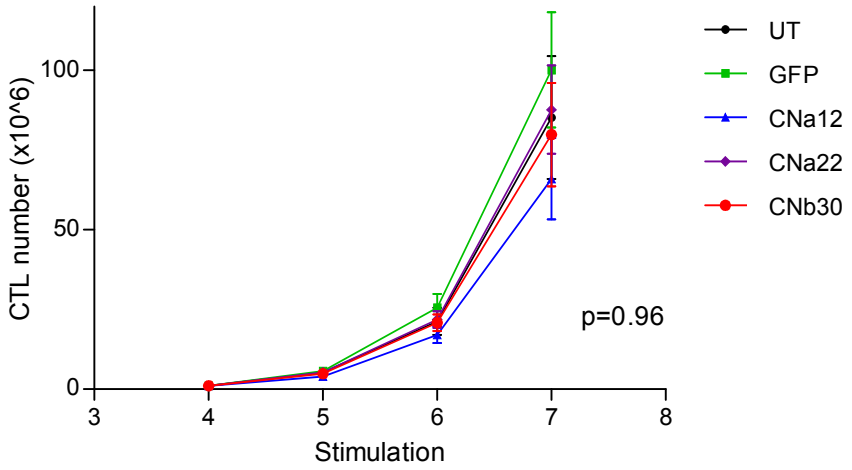


Figure 44: Comparable expansion of transduced and untransduced EBV-CTL lines in the absence of CN inhibitors. EBV-CTLs were transduced between stimulations 2 and 3, and proliferation monitored for 3 weeks from stimulation 4. No significant difference between the proliferation of UT and transduced CTLs was observed. Mean and SEM shown (*n=5*).

As discussed in section 4.2, CN is a pivotal molecule involved in activation of T cells, therefore raising the overall level of expression of CN by transduction with CN mutants, may have the potential to allow inappropriate proliferation of the transduced cell. Our data on CTL expansion in response to LCL stimulation confirm that EBV-CTLs transduced with CN mutants do not proliferate at a greater rate than UT EBV-CTLs. Next, proliferation of transduced and UT CTLs in the absence of stimulation was examined to determine if transduced CTLs can proliferate autonomously without T cell receptor stimulation. Transduced and UT CTLs were grown for four stimulations as normal, receiving weekly challenge with autologous LCL. LCL stimulation was subsequently withheld and CTL proliferation monitored, with or without continued supplementation with exogenous IL-2. As shown in Figure 45, neither UT nor EBV-CTLs transduced with SFG_CNb30_eGFP were able to proliferate without autologous LCL stimulation, even with continued addition of IL-2. No difference in proliferation was observed between transduced and UT EBV-CTLs when LCL stimulation was withheld (*p=0.994* with IL-2 supplementation, *p=0.991* with no IL-2, one-way ANOVA).

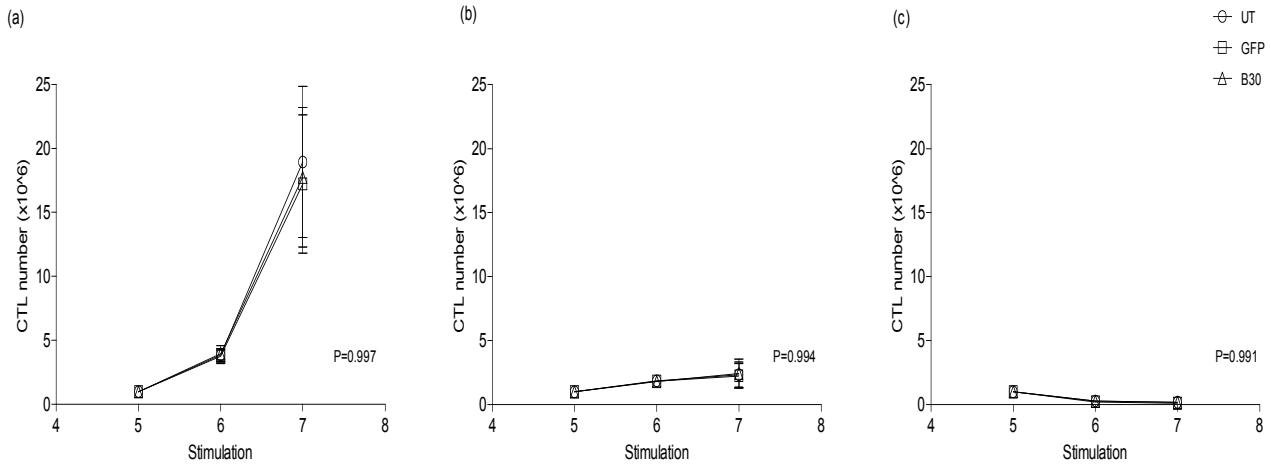


Figure 45: EBV-CTLs do not proliferate in the absence of LCL stimulation. (a) CTLs receiving stimulations 5 and 6 with autologous LCL expand as expected. No proliferation is observed from the same EBV-CTL lines not stimulated with autologous LCL either with (b) or without (c) continued addition of exogenous IL-2. No difference is seen between transduced and untransduced cell lines. Mean and SEM shown (*n=5*).

4.5.3 Transduction with CN mutants allows proliferation in the presence of FK506 or CsA

We next examined the ability of CN mutants to confer resistance to CN inhibitors. Transduction efficiencies of 60-80% were achieved for all lines and therefore no enrichment of the transduced CTLs by cell sorting was performed. Therapeutic concentrations of FK506 (10ng/ml) or CsA (200ng/ml) were added to CTL cultures at the 4th stimulation and cell expansion and expression of GFP was monitored at every stimulation thereafter.

4.5.3.1 CN mutant transduced CTLs show a selective growth advantage in the presence of CN inhibitors

It was anticipated that in the presence of CN inhibitors, cells transduced with mutants conferring resistance would continue to proliferate whereas untransduced cells would not. Therefore, within a transduced line the non-transduced cells would be suppressed whereas the transduced fraction would proliferate, leading to an enrichment of GFP positive cells. This was indeed observed: in the presence of FK506 the mean proportion of CTLs expressing GFP in CNa12 transduced cultures increased from 64 to 82% (*p=0.0018*) and in CNb30 transduced cultures from 71 to 90% (*p=0.01*). In the presence of CsA, GFP expression in CNa22 transduced cells rose from 63 to 76% (*n/s, p=0.1)* and in CNb30 transduced cultures from 71 to 91% (*p=0.01*, GFP expression compared between stimulations 4 and 7, all assessed using paired t test). These data indicate that transduction with our selected CN mutants confers a selective growth advantage in the presence of CN inhibitors. In contrast, expression of GFP in CTL lines grown with no immunosuppression remained stable throughout the study period for GFP, CNa12 and CNb30 transduced cells, indicating that transduced CTLs do not have an intrinsic growth advantage over non-transduced cells. However, a drop in the number of GFP expressing cells was observed in CNa22 transduced CTL lines in the absence of immunosuppression (see Figure 46). Interestingly, mutant CNa22 allowed the highest level of IL-2 secretion from Jurkat cells, and also the highest degree of hyper-activation upon stimulation in our previous cell line work. In line with the suggestion that proliferation of EBV-CTLs transduced with bicistonic constructs is poor due to the induction of activation induced cell death, it is possible that transduction with this active CN mutant also leads to induction of activation induced cell death in primary EBV-CTLs. This would result in the observed reduction in proliferation and gradual drop in expression of CNa22 where there is no selective advantage due to the presence of CsA.

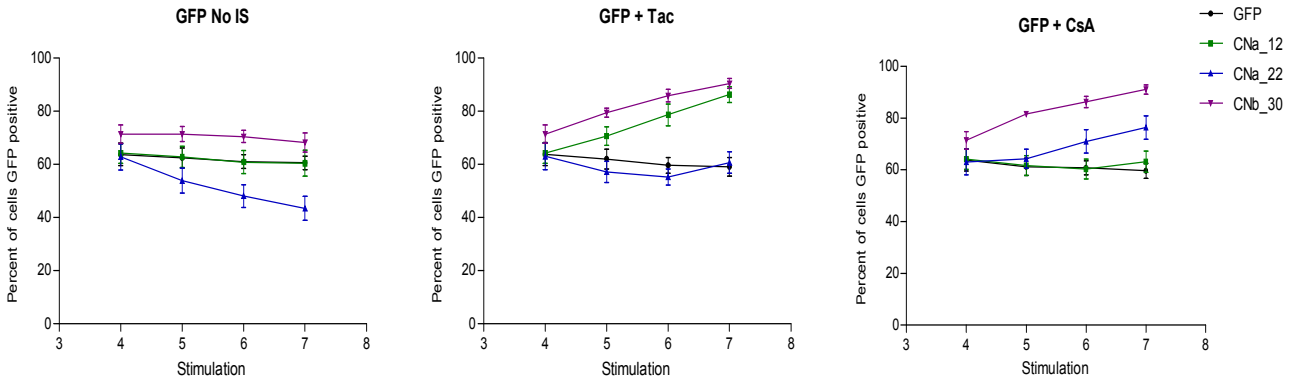


Figure 46: GFP expression in transduced EBV-CTL lines grown in the presence or absence of CN inhibitors. GFP expression was monitored by flow cytometry at each stimulation for all CTL lines (a) in the absence of CN inhibitors, (b) when grown with 10ng/ml FK506, or (c) when grown in 200ng/ml CsA. Mean and SEM shown (*n=5*).

4.5.3.2 Transduction of EBV-CTLs with CN mutants allows proliferation in the presence of CN inhibitors

Proliferation of transduced and UT EBV-CTL lines in the presence or absence of CN inhibitors was monitored from stimulation four to seven. An 85 fold expansion of UT EBV-CTLs was observed in this time in the absence of CN inhibitors. Addition of either 10ng/ml FK506 or 200ng/ml CsA to EBV-CTLs markedly inhibited growth of both untransduced and GFP transduced lines (Figure 47(i) and (ii), *p=0.001*, one-way ANOVA comparing expansion in absence of IS to expansion with FK506/CsA).

In contrast, as shown in Figure 47(iii), CNa12 transduced CTLs were able to expand in the presence of FK506 (*p=0.216*) but not CsA (*p=0.001*). CNa22 transduced CTLs grew in the presence of CsA (*p=>0.999*) but not FK506 (*p=0.004*) (Figure 47(iv)) and CNb30 transduced CTLs were able to proliferate in the presence of either CN inhibitor (FK506 *p=0.649*, CsA *p=>0.999*, all assessed by one-way ANOVA compared to expansion of UT CTL in absence of IS. See Figure 47(v)). When compared to 85-fold expansion of UT CTLs in the absence of immunosuppression, expansion of CNa12 CTLs in FK506 was 53% (44 fold), expansion of CNa22 CTLs in CsA was 106% (90 fold) and proliferation of CNb30 CTLs was both 67% in FK506 (57 fold) and 88% in CsA (75 fold).

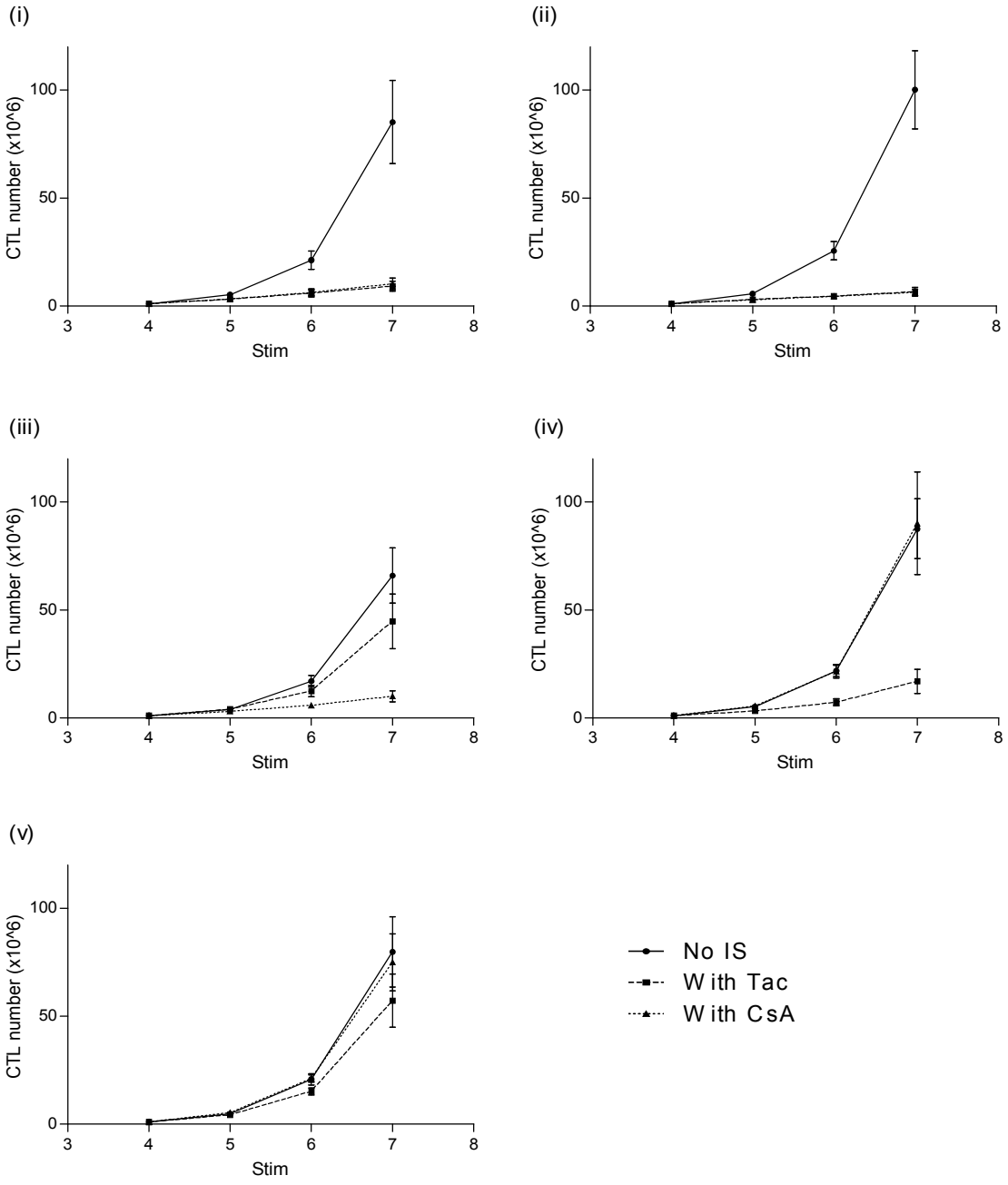


Figure 47: Transduction of EBV-CTLs with CN mutants allows proliferation in the presence of FK506/CsA. EBV-CTLs were transduced after stimulation 2. CN inhibitors were added at stimulation 4 and CTL growth was monitored for 3 weeks (Mean and SEM shown, *n=5*). (i) Untransduced CTLs are suppressed by either FK506 or by CsA. (ii) GFP transduced CTLs are suppressed by either CN inhibitor. (iii) CNa12 transduced CTLs proliferate when grown in FK506 but not CsA. (iv) CNa22 transduced CTLs proliferate in CsA but not FK506. (v) CNb30 transduced CTLs proliferate in either FK506 or CsA.

These results were confirmed by performing ^{3}H-thymidine uptake assays after stimulation 6, following three weeks growth in CN inhibitors. In the absence of CN inhibitors, proliferation of all transduced CTLs was comparable to UT CTLs (*p=0.876*, one-way ANOVA). As shown in Figure 48, the observed resistance profile in this assay was similar to that seen above. Untransduced or GFP transduced CTLs showed reduced proliferation in the presence of either 10ng/ml FK506 or 200ng/ml CsA, CNa12 transduced CTLs were able to proliferate in the presence of FK506 but not CsA and CNa22 showed the converse pattern. Once again, transduction with CNb30 enabled CTL proliferation in the presence of either FK506 or CsA. Compared to proliferation of UT CTLs in the absence of immunosuppression, CNa12 CTLs proliferated 86% in FK506 (*p=>0.999*), CNa22 CTLs proliferated 103% in CsA (*p=>0.999*) and CNb30 CTLs proliferated both 83% in FK506 (*p=0.996*) and 86% in CsA (*p=0.999*, all assessed by one-way ANOVA).

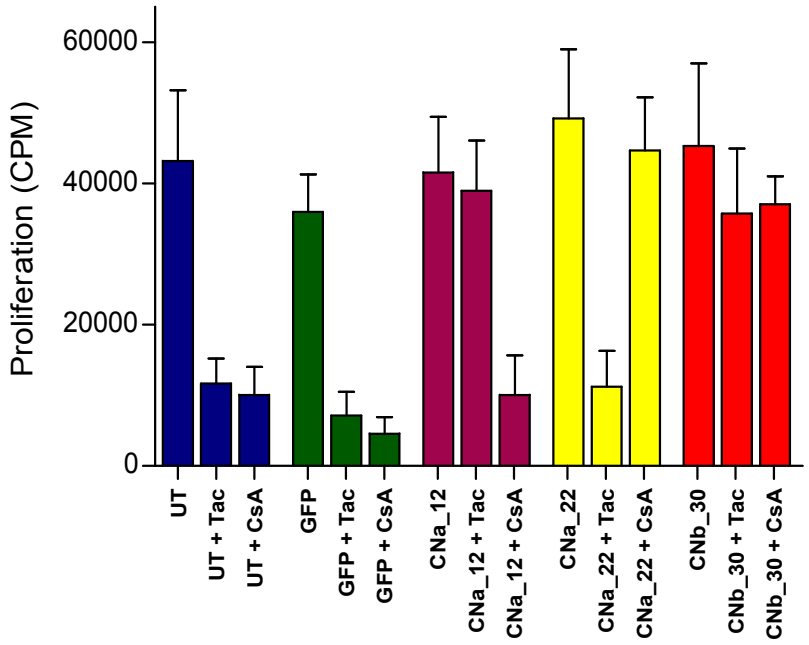


Figure 48: Transduction with CN mutants allows ^{3}H-thymidine uptake in the presence of FK506/CsA. A ^{3}H-thymidine uptake assay was performed following three weeks of growth in CN inhibitors. Mean and SEM shown *(n=5)*.

Furthermore, the ability of CNb30 transduced EBV-CTLs to proliferate in supratherapeutic concentrations of CN inhibitors was assessed in two donors. UT, GFP or CNb30 transduced CTLs were cultured for two weeks in 40ng/ml FK506 or 800ng/ml CsA, a four fold increase compared to the normal target dose. Once again, proliferation was assessed by ^{3}H-thymidine uptake assay and these results confirmed that CNb30 transduced EBV-CTLs are capable of proliferation in increased doses of CN inhibitors (see Figure 49). Compared to UT CTL proliferation without CN inhibitors, CNb30 transduced CTL proliferation from two cell lines was 117% in FK506 and 60% in CsA.

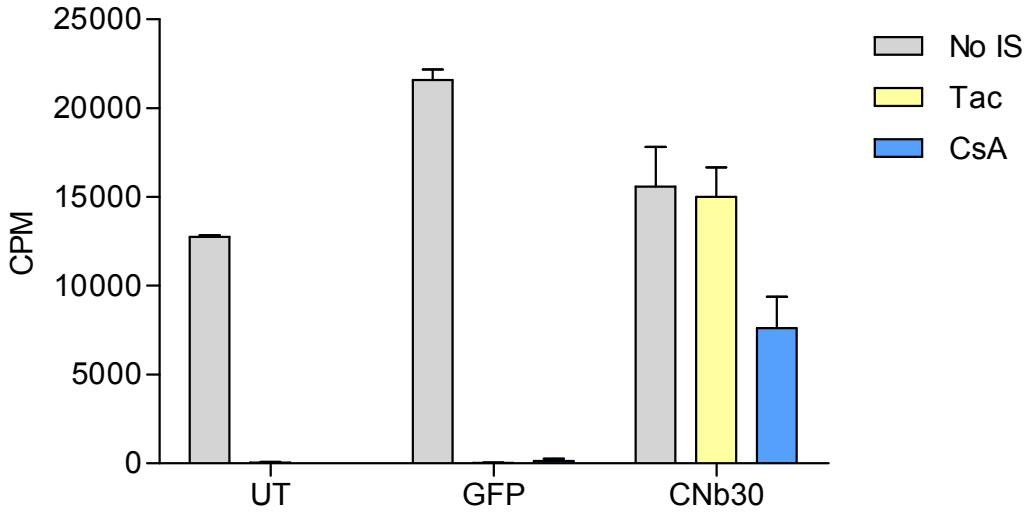


Figure 49: Transduction with CNb30 allows proliferation in supratherapeutic doses of FK506/CsA. Proliferation of CTLs was assessed by thymidine uptake following two weeks of culture in the presence of supratherapeutic concentrations of CN inhibitors. FK506 40ng/ml, CsA 800ng/ml. Mean and SEM shown (*n=2*).

These results confirm that transduction of EBV-CTLs with CN mutants confers resistance to suppression of proliferation by CN inhibitors. We have generated one mutant that confers resistance to FK506 but not CsA (CNa12), one that confers resistance to CsA but not FK506 (CNa22) and one that confers resistance to both CN inhibitors (CNb30).

4.5.4 Transduction with CN mutants allows secretion of IFN-γ in the presence of FK506 or CsA

To further examine the functionality of EBV-CTL lines transduced with our CN mutants, the ability of transduced CTLs to secrete cytokines in the presence of immunosuppressive agents was assessed. As shown previously, EBV-CTLs produce high levels of IFN-γ in response to stimulation with autologous LCL and we confirmed that IFN-γ secretion from transduced cells in the absence of CN inhibitors is comparable to that from UT EBV-CTLs (*p=0.984*, one-way ANOVA). However this is abrogated by the addition of either FK506 or CsA. Following two weeks growth in CN inhibitors, culture supernatant was harvested for analysis of IFN-γ secretion by ELISA.

IFN-γ secretion by UT or GFP transduced EBV-CTLs was abrogated by 10ng/ml FK506 or 200ng/ml CsA. In contrast, as shown in Figure 50, EBV-CTLs transduced with CNa12 were able to secrete IFN-γ in the presence of FK506 (mean 66% secretion compared to untransduced CTLs in the absence of CN inhibitors (*p=0.914*)), but not CsA (mean 17% secretion, *p=0.062*). CNa22 transduced CTLs secreted IFN-γ in the presence of CsA (mean 66%, *p=0.917*), but not FK506 (mean 16%, *p=0.059*). As expected, CTLs transduced with CNb30 were able to secrete IFN-γ in the presence of either FK506 (mean 92%, *p=>0.999*) or CsA (mean 100%, *p=>0.999*, one-way ANOVA).

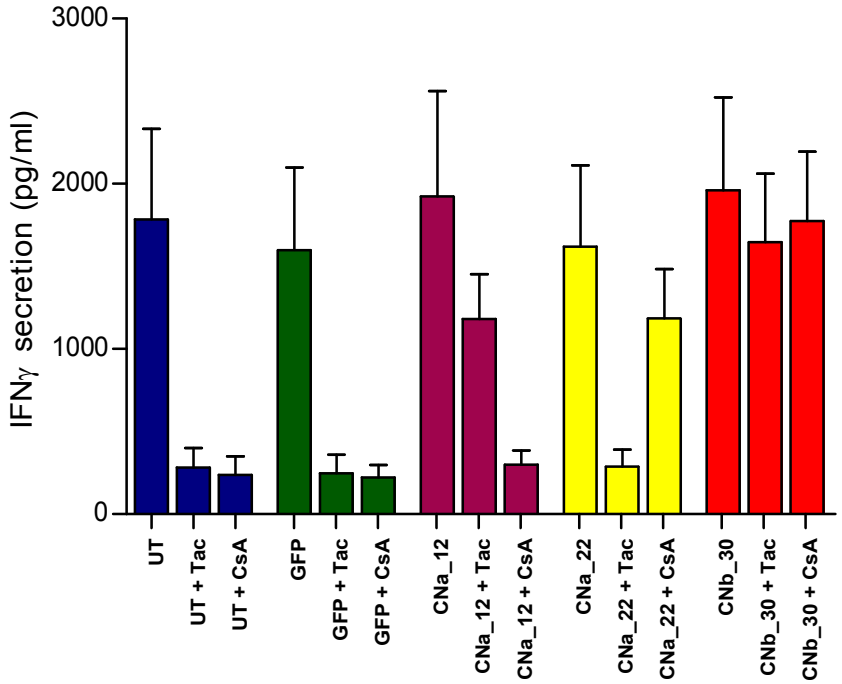


Figure 50: CN transduced EBV-CTLs secrete IFN-γ in the presence of FK506/CsA. Transduced and UT EBV-CTLs were grown in the presence or absence of FK506/CsA for 2 weeks. IFN-γ secretion was measured by ELISA 24 hours following the 6th stimulation. Mean and SEM shown (*n=5*).

4.5.5 Effect of transduction on EBV-CTL cytotoxicity

As discussed in section 4.2, discrepancies are present in the published literature regarding the effect of CN inhibitors on CTL cytotoxicity. Therefore, to determine the effect of transduction with either GFP alone or CNb30 on the cytotoxicity of EBV-CTLs, killing of LCL targets by transduced and untransduced EBV-CTLs was examined. Subsequently, the effect of addition of CN inhibitors to the culture both prior to and during the cytotoxicity assay was examined.

These results demonstrated that transduction of EBV-CTLs with either GFP alone or CNb30 had no effect on cytotoxicity towards autologous LCL, allogeneic LCL or HSB-2 target cells (Figure 51, *p=0.821*, all groups compared by one-way ANOVA). Specific killing of autologous LCL of between 25 and 30%, 5-7% killing of allogeneic LCL and 22-26% killing of HSB-2 cells was observed by both UT and transduced

CTLs for all lines. The modest level of cytotoxicity against autologous LCL may reflect the early transduction procedure which was performed between stimulations 2 and 3 in order to complete the planned assays prior to the expected proliferation plateau after stimulation 7. Likewise, the early addition of high concentrations of IL-2 (100U/ml) to these CTL lines is likely to contribute to higher non-specific kill of HSB-2 targets. Nonetheless, it is clear from these experiments that cytotoxicity towards all targets was not affected by transduction with either GFP or CNb30.

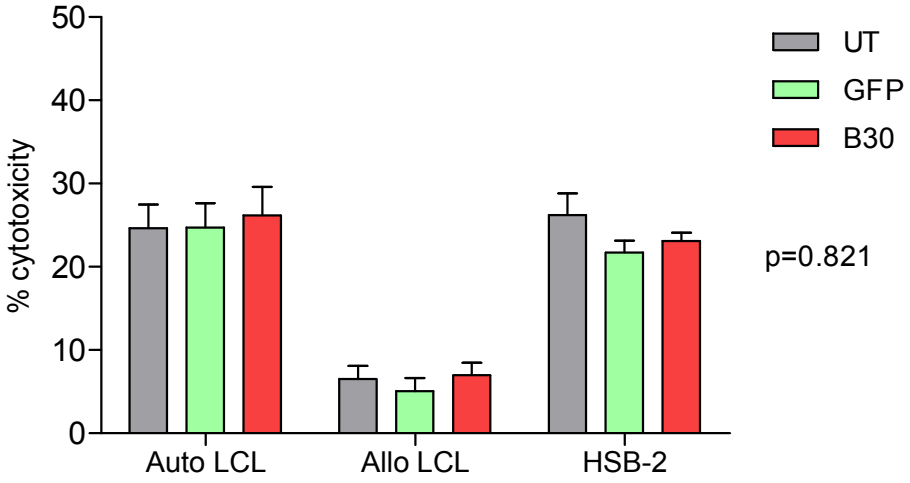


Figure 51: Cytotoxicity of EBV-CTLs is unaffected by transduction with CNb30 or GFP. 4 hour ^{51}Chromium release killing assay was performed after stimulation 5 with autologous LCL, allogeneic LCL and HSB-2 targets at an E:T ratio of 30:1. Mean and SEM shown (*n=5*).

4.5.6 Effect of CN inhibitors on EBV-CTL cytotoxicity

To determine the effect of CN inhibitors on cytotoxicity of EBV-CTLs, 10ng/ml FK506 or 200ng/ml CsA were added to culture conditions at stimulation 4. Cytotoxicity assays were performed on the day of the 6th or 7th stimulation, when cells had been growing for either two or three weeks in these CN inhibitors. FK506 or CsA were also included in the cytotoxicity assay. As shown in Figure 52, in our hands, prior growth in CN inhibitors and addition of these to the cytotoxicity assay had no effect on killing of target cells by EBV-CTLs. Lysis of autologous LCL was 24-34% by both transduced and UT CTLs and was comparable with or without CN inhibitors. There were no significant differences as a result of CN inhibitor addition (UT *p=0.841*, GFP *p=0.728* and CNb30 *p=0.9*, both transduction and addition of IS compared by two-way ANOVA). Lysis of allogeneic LCL was 3-10% and lysis of HSB-2 cells was 22-33% for all conditions.

These data confirm that cytotoxicity of EBV-CTLs is not affected by transduction with CN mutants. Furthermore, in a standard 4 hour chromium release assay, no difference was detected in cytotoxicity of CTL lines as a result of CN inhibitor addition.

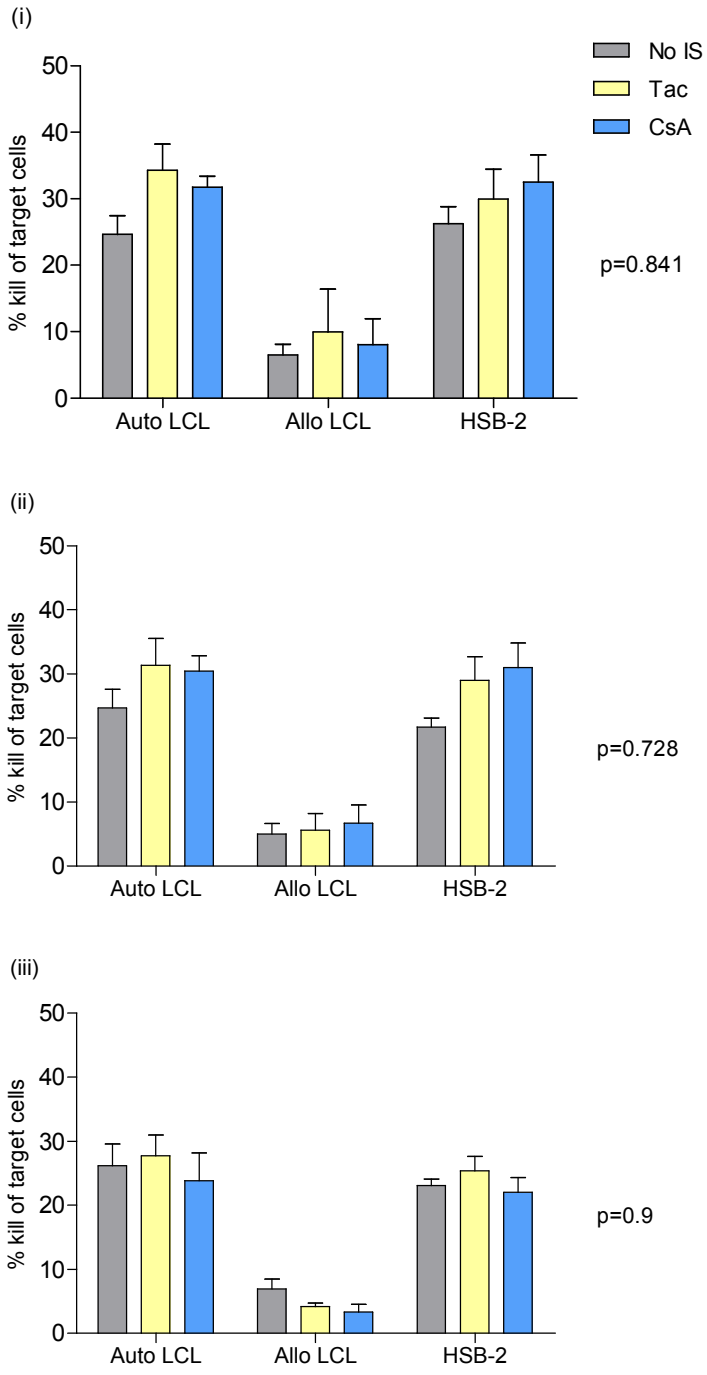


Figure 52: Addition of CN inhibitors does not affect cytotoxicity of transduced or untransduced EBV-CTLs. (i) UT (ii) GFP transduced and (iii) CNb30 transduced EBV-CTLs were grown without CN inhibitors (grey bars), with 10ng/ml FK506 (yellow bars) or with 200ng/ml CsA (blue bars) for 2-3 weeks prior to performing a cytotoxicity assay against autologous LCL, allogeneic LCL and HSB-2 targets. Mean and SEM at an E:T ratio of 30:1 are shown ($n=5$).

4.6 Conclusions

We have established a protocol for the efficient generation and transduction of EBV-CTL lines with CN mutants. As previously reported, EBV-specific CTLs can routinely be generated from peripheral blood samples of healthy donors and transduced with a typical efficiency of 50-80% (Quintas-Cardama*, et al* 2007, Wilkie*, et al* 2004). CTLs are not adversely affected by this procedure and subsequently continue to proliferate as normal. Importantly, we have confirmed that transduction with CN mutants does not lead to hyperactivation in terms of increased proliferation or IFN-γ secretion from transduced cells, and that these lines remain dependent on stimulation with specific antigen for continued growth. This confirms that raised intracellular CN does not allow activation in the absence of TCR stimulation, or enhanced activation responses upon stimulation. No aberrant proliferation or cytokine secretion was observed from transduced EBV-CTLs.

Proliferation of UT EBV-CTL lines is inhibited by addition of either FK506 or CsA to cultures. In contrast, EBV-CTL lines transduced with our selected CN mutants retain the ability to proliferate in the presence of the appropriate CN inhibitor. We have tested three CN mutants and shown that CTLs transduced with CNa12 proliferate in the presence of FK506, those transduced with CNa22 proliferate despite the addition of CsA, and those transduced with CNb30 proliferate as normal with either CN inhibitor. This was demonstrated by monitoring cellular expansion in CN inhibitors over a three week culture period, and confirmed by performing a ^{3}H-thymidine uptake proliferation assay. This confirmed a selective growth advantage to transduced CTLs in the presence of CN inhibitors, leading to enrichment of transduced CTLs within cultures.

Furthermore, we have examined the functionality of these transduced EBV-CTLs to confirm that they retain the ability to secrete the important Th1 cytokine IFN-γ in the presence of CN inhibitors. UT EBV-CTLs secrete high levels of IFN-γ in response to stimulation with autologous LCL, but this secretion is sensitive to suppression with FK506/CsA and is abrogated by their addition. Analogous to our data on proliferation, we have shown that EBV-CTLs transduced with our CN mutants are also resistant to

suppression of IFN-γ secretion, and continue to secrete normal levels of this cytokine in the presence of FK506 (CNa12), CsA (CNa22), or both (CNb30).

Finally, we examined the effect both of CN transduction and addition of CN inhibitors on the cytotoxicity of EBV-CTLs. We have demonstrated that transduction with CNb30 has no effect on killing of autologous LCL, allogeneic LCLs or HSB-2 target cells, and also that the presence of CN inhibitors does not affect cytotoxicity. This is in contrast to some previously published studies regarding cytotoxicity (Zhan*, et al* 2003), however in our hands 2 weeks of prior culture in CN inhibitors and inclusion of these in the killing assay did not lead to any reduction in cytotoxicity for UT or transduced CTL lines. Previous studies of CTL cytotoxicity have identified both CN dependant and independent cytotoxicity in a murine CTL clone (Kataoka and Nagai 2000). This may reflect the use of both FasL and cytolytic granule killing pathways by CTLs. These mechanisms were further elucidated by He and Ostergaard, demonstrating differential regulation of early FasL expression, late FasL expression and perforin/granzyme degranulation in murine alloreactive CD8+ clones (He and Ostergaard 2007). Work by Rogers *et al* suggests that the mechanism for induction of FasL cytotoxicity in CTLs contains two synergistic pathways, one sensitive and one insensitive to CsA, but that overall cytotoxicity is not affected by the presence of CsA (Rogers*, et al* 1997). A detrimental effect of CN inhibitors on CTL cytotoxicity was not observed here, however it remains possible that alternative or more sensitive assays may detect such differences. Given the restoration of other tested CN dependent mechanisms such as proliferation and IFN-γ secretion in CN mutant transduced CTLs, even if this was the case it is likely that any suppression of cytotoxicity by CN inhibitors would also be circumvented.

Taken together, these data confirm that transduction of EBV-CTL lines with CN mutants can confer resistance to suppression by CN inhibitors. We show restoration of the cellular events that are dependent on intact CN signalling, including cell proliferation and secretion of IFN-γ. No significant difference was observed in comparison of transduced CTL proliferation in the presence of FK506/CsA to UT EBV-CTL proliferation in the absence of CN inhibitors. This was also the case for IFN-γ secretion: transduced CTLs secrete normal levels of IFN-γ in FK506/CsA when

compared to UT CTLs without CN inhibitors. Thus, we have generated EBV-CTLs that appear to function as normal despite the presence of therapeutic doses of FK506/CsA *in vitro*. Indeed, expression of CN mutants enabled CTLs to proliferate in levels of CN inhibitors well above the therapeutic range. This approach could represent a considerable advance for the prophylaxis/treatment of PTLD arising following solid organ transplantation, by enabling transfer of EBV-specific immunity without withdrawal of IS, and the ability to render T cells resistant to CN inhibitors has wider implications in the application of adoptive immunotherapy.

The generation of mutants that confer different profiles of resistance could be beneficial in a clinical setting. EBV-CTLs that are resistant to one CN inhibitor remain sensitive to suppression with the alternative should an adverse event occur. This would allow temporary control rather than permanent deletion of infused cells. For example, inflammatory reactions have been documented at sites of bulky disease following immunotherapy and such events could be controlled by administration of the CN inhibitor the CTLs are not resistant to. Conversely, administration of CNb30 transduced EBV-CTLs allows the clinician to choose their preferred CN inhibitor with no negative influence on the efficacy of CTL therapy. An additional benefit of CNb30 over CNa12/22 with regard to vector design is its small size. At approximately 500bp, CNb30 could easily be co-expressed with additional genes that may be desirable. For example, expression of a suicide gene such as iCasp9 may be required to allay safety concerns, a cytokine gene (IL-2/IL-15) to enhance function, or a conventional or chimaeric T cell receptor to re-direct EBV-CTLs towards tumour antigens are possible combinatorial applications.

In light of these considerations, CNb30 was pursued as our preferred mutant in studies using a small animal model to demonstrate functionality in the presence of therapeutic doses of CN inhibitors *in vivo*.

4.7 General conclusions

- EBV-CTL lines generated from peripheral blood samples of healthy donors can be efficiently transduced with RV vectors containing CN mutants.
- Stably transduced retrovirus producer lines encoding CNa12, CNa22, CNb30 and GFP have been generated.
- Transduction of EBV-CTL lines with CN mutants was shown to have no effect on phenotype or function in the absence of CN inhibitors.
- Transduced EBV-CTL lines are rendered resistant to FK506, CsA or both CN inhibitors enabling them to proliferate, secrete IFN-γ and lyse EBV infected targets in the presence of these drugs.

Chapter five

Establishing an *in vivo* model for evaluating CN inhibitor resistant EBV-CTLs

5.1 Aims

- To establish a murine model of PTLD development.
- To establish and optimise a Luciferase imaging system for monitoring tumour development.
- To examine the efficacy of untransduced EBV-CTLs as treatment and prophylaxis for PTLD *in vivo.*
- To assess the effect of CN inhibitors on untransduced or CNb30 transduced EBV-CTL activity *in vivo.*

5.2 Introduction

Following demonstration that EBV-CTLs transduced with CN mutants retain their function *in vitro* in the presence of CN inhibitors, we wished to demonstrate whether this approach would enable CTLs to function in the face of these drugs *in vivo* prior to scale up and planning of clinical trials. As discussed in section 1.6.2, the SCID mouse model of PTLD has been widely used to study PTLD development and treatment and was therefore utilised for this purpose. Lacerda *et al* have shown that either i.p. or i.v. administration of autologous EBV-CTLs resulted in either delayed progression or complete regression of tumours (Lacerda*, et al* 1996). This model has also been employed by several other groups to examine anti-tumour CTL activity (Foster*, et al* 2008, Savoldo*, et al* 2007).

Our department has previously generated and maintains a breeding colony of triple knockout γ-chain$^{-/-}$/RAG2$^{-/-}$/C5$^{-/-}$ SCID mice (3KO) which were used for these investigations. The 3KO mouse was produced to provide an appropriate model for studying reconstitution of the haematopoietic system. The common γ-chain is integral

in cytokine signalling through IL-2, 4, 7, 9 and 15 and plays an important role in lymphoid development, the absence of which in humans results in X-linked severe combined immunodeficiency. The murine homozygous mutant γ-chain phenotype results in both abnormal lymphopoiesis and dysfunction of residual T cells. Importantly, likely due to absent IL-15 signalling, γ-chain knockout mice lack functional NK cells, the activity of which poses a barrier to modelling immunotherapy. The absence of RAG2 recombinase activity prevents rearrangement of TCR and BCR resulting in a lack of T and B lymphocytes. Therefore double knockout mice have no T, B or NK cells (Goldman*, et al* 1998). In addition, back-crossing of these mice onto the naturally C5 deficient A/J strain results in the 3KO model, with additional defects in innate immunity which enhances human cell engraftment (M. Blundell, personal communication). 3KO are entirely devoid of T, B and NK cells, therefore these animals must be maintained in individually ventilated cages (IVCs) with sterile air, food, water and bedding and handled under sterile conditions. As this strain lacks T, B and NK cells it is able to accept human xenografts. Therefore in contrast to CB17 SCID mice, no NK depletion is required prior to CTL infusion (Wagar*, et al* 2000). We felt that this represented a significant advantage over the Lacerda model and therefore have developed a similar PTLD model in 3KO mice to allow *in vivo* testing of CNb30 transduced EBV-CTL activity.

In addition we have established and optimised bioluminescent imaging using the Xenogen IVIS system to monitor tumour development. Physical measurement of tumours using callipers has been the end point for previous models of PTLD, however this is a subjective method with the potential for considerable inter-operator variation and bias. Bioluminescent imaging utilising Luciferase expression represents a powerful method by which cells can be tracked or monitored, the use of which is rapidly increasing. Several recent studies have used bioluminescence to monitor both tumour cells and adoptively transferred T cells (Foster*, et al* 2008, Savoldo*, et al* 2007), and we have used this method to assess tumour progression in our model.

In order to examine the effect of CN mutant transduction on EBV-CTL efficacy *in vivo*, correct dosing to achieve therapeutic levels of CN inhibitors in the animals is necessary. Various routes, doses and formulations of administration have been utilised

in the published literature, including i.p. injection, i.v. injection, gavage and osmotic pumps (Lopes*, et al* 2008, McAlister*, et al* 1999, Medyouf*, et al* 2007, Yang*, et al* 2002). These publications have generally studied the effect of a particular dose of drug without confirming the resulting circulatory concentration. In contrast, we have attempted to determine the appropriate dose required to produce a therapeutic blood concentration.

In order to progress towards treatment of PTLD patients with CN mutant transduced CTLs, testing of this protocol *in vivo* is an essential step. Establishment of the PTLD model, treatment of these tumours with EBV-CTLs, imaging and dosing of the animals with CN inhibitors are all critical elements of this process. We have attempted to optimise each of these elements in our studies below.

5.3 Establishing murine model of PTLD development

5.3.1 Subcutaneous engraftment of human LCL in 3KO mice

To allow accurate planning of subsequent experiments, reliable development of tumours at a consistent time point is desirable. LCL from six donors were examined for the ability to produce tumours in SCID mice. 10^7 LCL in matrigel were injected s.c. and the animals monitored three times weekly for up to 8 weeks. Three 3KO mice were evaluated for each line to assess the consistency of kinetics of tumour development. This resulted in the detection of palpable tumours generated from the LCL of all but one donor (see Table 13).

Table 13: Development of subcutaneous LCL tumours from 5 of 6 donors

Donor	Number of animals	Tumour development?	Average time (days)	Range
A	3	Yes	21	20-22
B	2	No	-	-
C	2	Yes	10	10
D	2	Yes	13	10-16
E	3	Yes	7	7
F	2	Yes	10	10

These results show that in 5 evaluable LCL lines, development of subcutaneous tumours is both reliable and showed consistent kinetics, with tumours from the same donor appearing in different animals within a window of a few days. Tumour samples were sectioned and stained. Haematoxylin and eosin (H&E) stains (Figure 53a) showed the tumour to be histologically similar to human PTLD with the appearance of a diffuse large B-cell lymphoma (work performed by Dr. Neil Sebire, consultant histopathologist, GOSH). Staining with a monoclonal antibody reactive to human CD20 showed that the tumour is composed of human CD20 positive B cells, confirming that the tumour develops from the subcutaneously injected human LCL (Figure 53b).

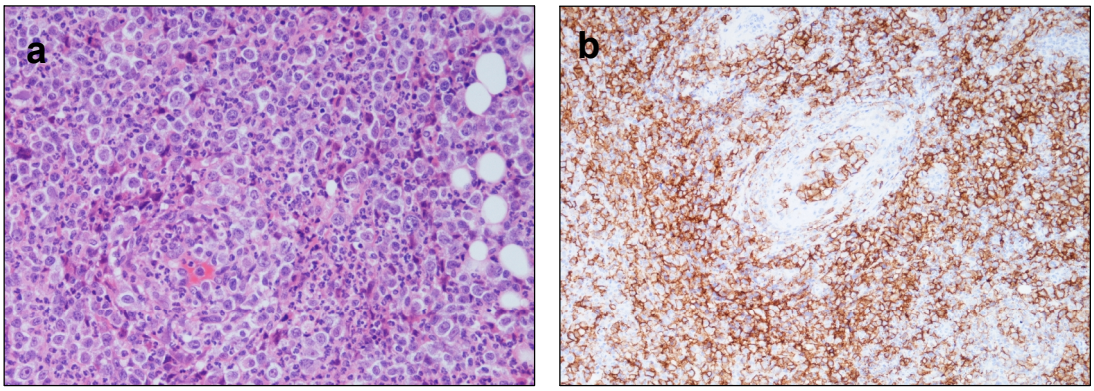


Figure 53: Staining of excised subcutaneous tumour demonstrates LCL origin. Tumour excised from SCID mouse 10 days after LCL injection. (a) 200x magnification of H&E stain. (b) 100x magnification of human CD20 monoclonal antibody staining.

5.4 Luciferase imaging of tumours *in vivo*

Following confirmation that subcutaneous injection of LCL induces formation of B cell tumours in 3KO mice, we focussed on optimising tumour imaging using bioluminescence. Using the IVIS imaging system to detect light output following intraperitoneal (i.p.) injection of D-Luciferin, the amount of Luciferase expression can be measured as a surrogate for tumour size.

5.4.1 Transduction of LCL with FLuc

LCL from three donors were transduced with an SFG retroviral vector encoding green-shifted Firefly Luciferase (FLuc), and one week after transduction, LCL were FACS sorted based on expression of the eBFP reporter gene, to select for transduced cells. Figure 54 shows FLuc-eBFP transduction and FACS sorting of three donor LCL lines. 10^7 sorted FLuc transduced LCL were injected subcutaneously in matrigel into 3KO animals to optimise imaging of the LCL population *in vivo*.

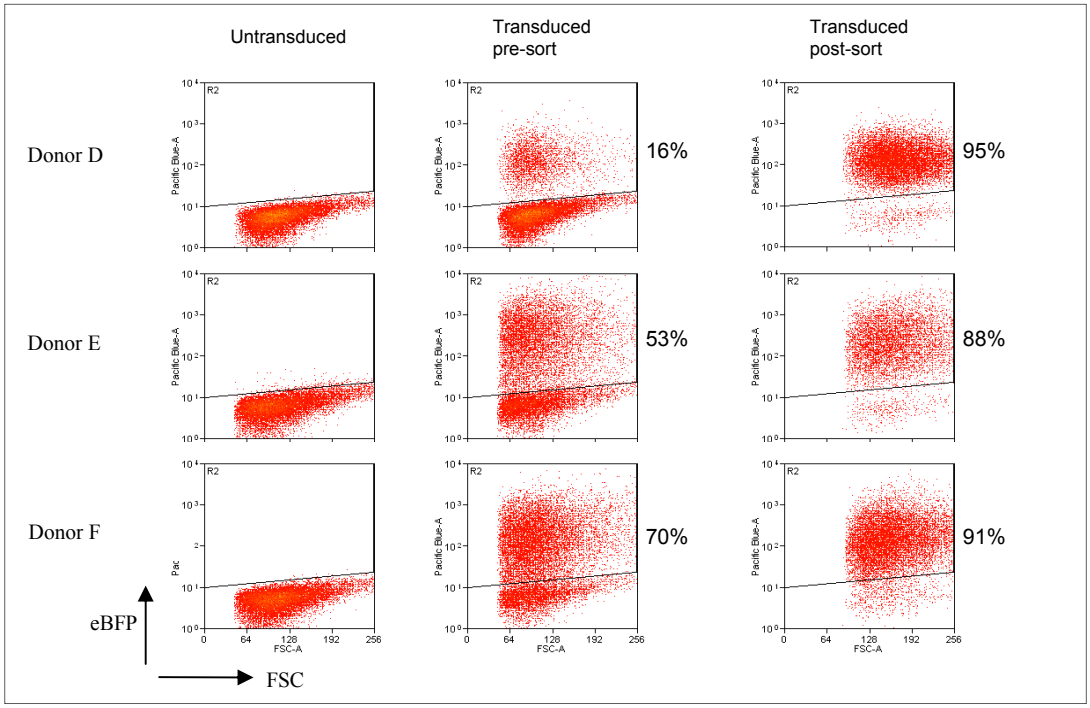


Figure 54: Transduction of LCL lines from 3 donors with FLuc. Expression of eBFP was assessed before and after FACS sorting.

Green-shifted FLuc contains mutations at the following positions: V241I/G246A/F250S (Branchini*, et al* 2007), which may facilitate conformational changes of the enzyme and in addition may increase the hydrophobicity of the emitter site, resulting in light emission at 544nm rather than 558nm. This is advantageous as it can be combined with red-shifted FLuc which emits at 612nm and the two enzymes imaged together with green and red filters, therefore two cell populations can be imaged following injection of a single substrate. In contrast, transduction of one cell population with FLuc and another with RLuc requires administration of two different substrates (D-Luciferin and coelenterazine respectively) and separate imaging. Thus, potentially the use of red and green-shifted FLuc should allow single substrate imaging and detection of both transduced LCLs and CTLs in the future. However, for our work to date, a single population of green-FLuc transduced LCL was imaged.

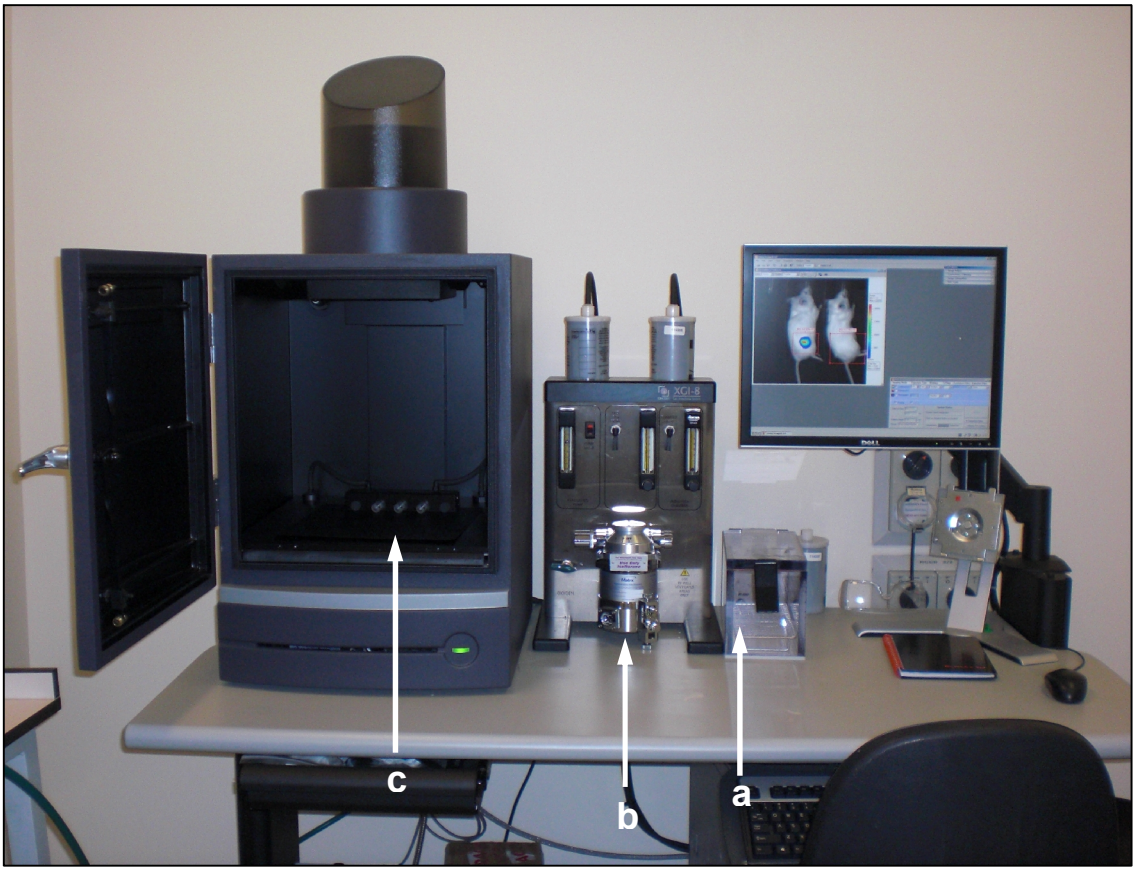


Figure 55: IVIS imaging system. (a) Induction chamber, (b) anaesthesia control and (c) imaging chamber are indicated.

5.4.2 Imaging in IVIS system

To carry out imaging of mice injected with FLuc expressing cells, anaesthesia is induced by inhalation of 2% isofluorane and oxygen in the induction chamber. The imaging chamber contains three outlets to maintain anaesthesia during imaging (see Figure 55). The terms of project licence PPL70_5249, under which these investigations are carried out, stipulates that animals can be monitored in this way for up to 60 minutes before recovery individually in warmed chambers.

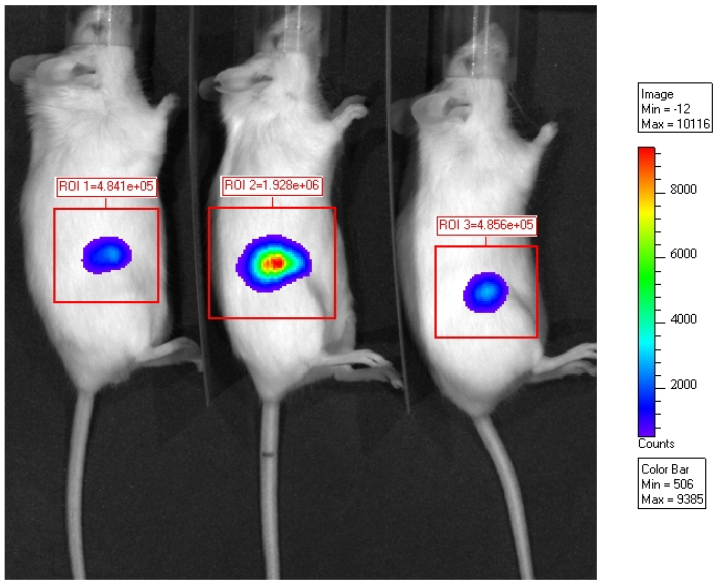


Figure 56: Example of imaging animals using IVIS system. Three 3KO mice were injected subcutaneously with 10^7 FLuc transduced LCL and bioluminescent imaging performed after 12 days. The region of interest was identified around each tumour and the light emission from that area quantified.

An example of output from the IVIS imaging system is shown in Figure 56. A light photograph of the animals is captured and merged with the light emission image to allow identification of the area of interest. A region of interest (ROI) is delineated around the tumour, and the number of counts per second emitted from that area can be calculated. As a result of high FLuc expression in sorted LCL, the brightness of signal from FLuc and low light emission from normal tissues, negligible background and clear signal regions can be identified in these images.

5.4.3 Timing of D-luciferin injection

Following injection of the Luciferase substrate into the peritoneum of an animal, D-luciferin is absorbed into the circulation and transported to the site of the tumour where it is catalysed by Luciferase to produce light. However, in addition to this, luciferin is metabolised by the liver and excreted by the kidneys of the mouse (Berger, *et al* 2008b). Therefore, following an increase in signal as Luciferin is absorbed and

the circulatory concentration gradually rises, the substrate is metabolised, blood concentration drops and the light emitted plateaus then declines. Breakdown of D-luciferin is therefore affected by the metabolic rate of the mouse which introduces additional practical considerations for imaging. As can be seen in Figure 57a, injection of D-luciferin into anaesthetised mice leads to a gradual rise in signal, which then plateaus approximately 25-35 minutes following injection. In contrast, injection of substrate into a conscious mouse followed by induction of anaesthesia and imaging leads to a much more rapid rise, plateau and fall in signal, such that the plateau has passed upon imaging at 15 minutes (Figure 57b), reflecting the increased metabolic rate when the mouse is conscious.

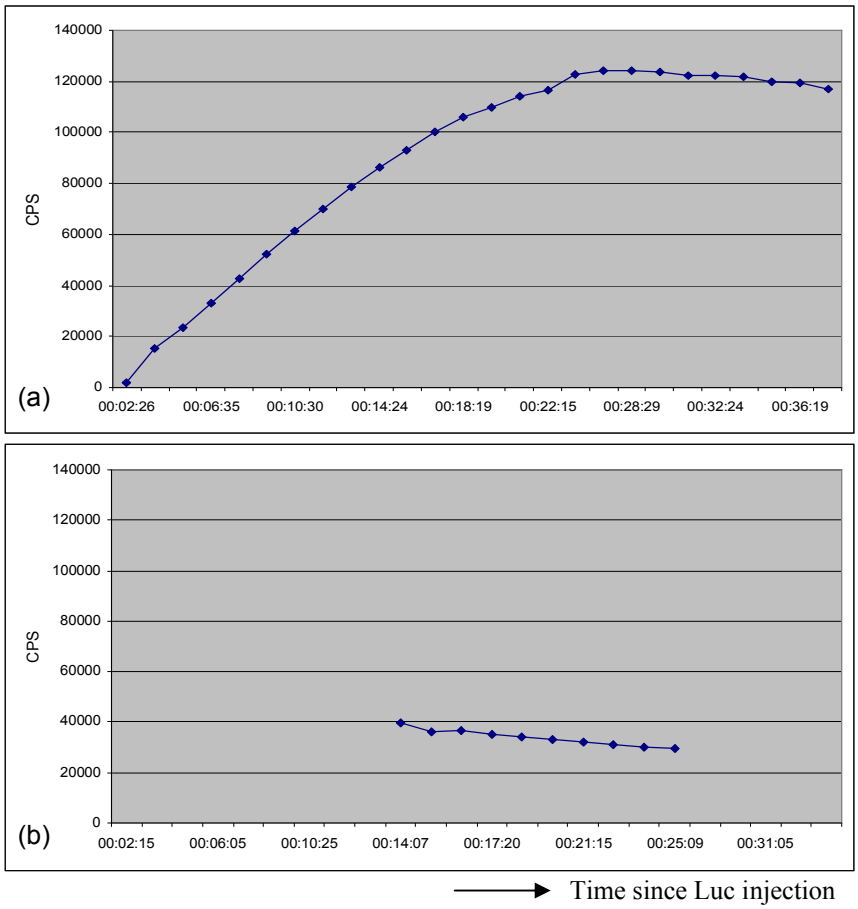


Figure 57: Kinetics of FLuc signal following substrate administration. The same animal was imaged after administration when (a) anaesthetised or (b) awake. When anaesthetised, signal rises for 25 minutes, plateaus for approximately 10 minutes and drops slowly. When awake, plateau occurs prior to imaging from 14 minutes onwards.

These data highlight the need for consistency of protocol. Administration of D-luciferin substrate following anaesthesia allows monitoring of the rise, plateau and fall of signal as the substrate is absorbed and metabolised. Although the time of anaesthesia is increased using this method, consistent results can be reliably obtained therefore this protocol was used throughout subsequent experiments.

5.4.4 Consistent expression of Luciferase from LCL

When monitoring biological processes based on expression of a transgene such as Luciferase, an important consideration is the continued and stable expression of the reporter gene. Fluctuations in the level of FLuc expression from LCL *in vivo* would result in signal changes and be interpreted as changes in tumour size; therefore it is necessary to determine that expression of FLuc is not silenced and that expression remains stable over the experimental period *in vivo*. Therefore, expression of FLuc from transduced LCL was assessed prior to subcutaneous injection. Following 3½ weeks of growth *in vivo* the resulting tumour was excised and expression examined again (see Figure 58). Prior to administration, 85% of LCL expressed eBFP. Following excision, the tumour sample was homogenised and stained with anti-human CD45 and CD19 antibodies before analysis by flow cytometry. 83-85% of cells expressing either human CD45 or human CD19 from the tumour sample remained eBFP positive. These results demonstrated that during 3½ weeks *in vivo*, silencing of the transgene was not observed.

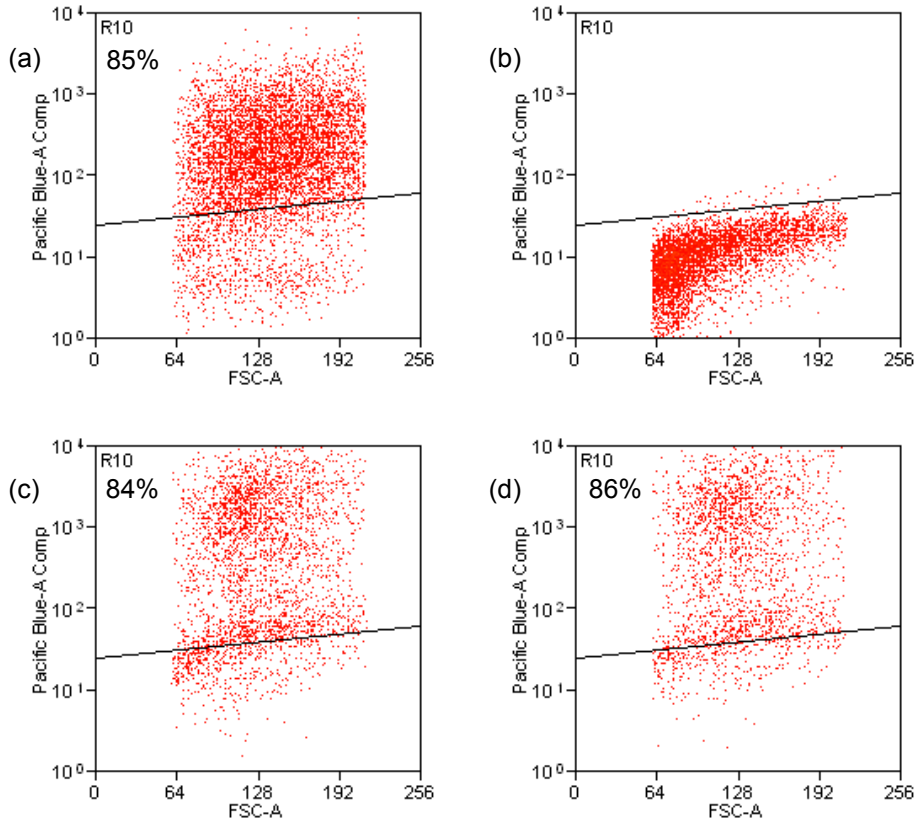


Figure 58: Expression of FLuc remains stable over 3.5 weeks *in vivo*. Analysis of eBFP reporter gene expression in (a) LCL line prior to injection. (b) Blood sample from mouse after 3½ weeks bearing LCL tumour. (c,d) excised tumour sample gated on cells expressing human CD19 or CD45 from two different mice.

5.5 Treatment of EBV-PTLD with EBV-CTLs

5.5.1 EBV-CTL induced regression of LCL tumours

A pilot experiment was carried out to establish whether i.v. injection of autologous EBV-CTLs from three donors resulted in regression of LCL tumours *in vivo*. Three donors of the five whose LCL led to tumour formation in previous experiments were selected. Cryopreserved EBV-CTLs frozen after two stimulations were available for injection from these donors. Three mice per donor received 10^7 LCL s.c. and were monitored for the presence of palpable tumour, whereupon the animals were imaged and 10^7 viable autologous EBV-CTLs were thawed and injected i.v. into two of the

animals, the third remaining untreated. This dose of CTLs was chosen on the basis of that used by Lacerda *et al* in previous studies (Lacerda, *et al* 1996). Following CTL administration, all animals received 2500U recombinant human IL-2 i.p. three times weekly. The animals were monitored biweekly with bioluminescence imaging and three times weekly with calliper measurement of the tumour to determine tumour progression. Imaging results were correlated with tumour size to confirm the accuracy of tumour monitoring using bioluminescence.

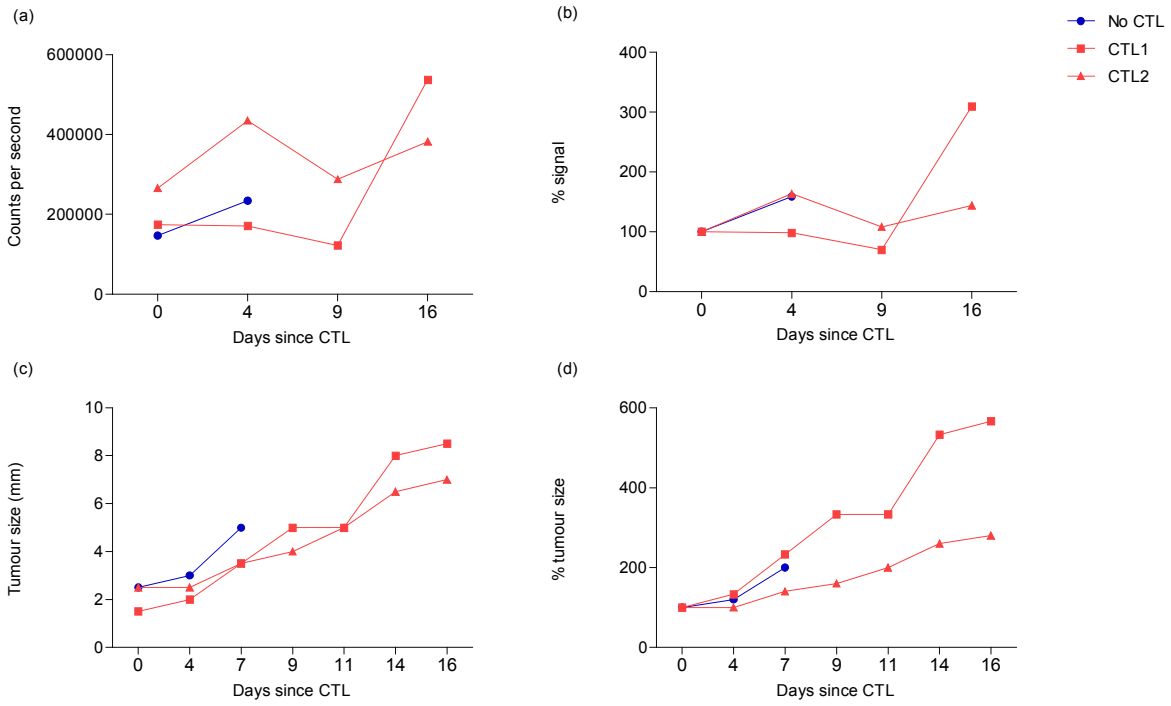


Figure 59: Bioluminescence and size monitoring of EBV-CTL treated and untreated tumours established from Donor E LCL. CTLs were injected into treated mice (red lines) on day 0. (a) Absolute light emission from ROI. (b) Light emission relative to signal on day of CTL injection. (c) Tumour size measured in mm. (d) Tumour size relative to size on day of CTL injection.

In our initial experiment, shown in Figure 59, the effect of administration of EBV-CTLs on the growth of LCL tumours from donor E could not be established. The

tumour carried on the untreated animal doubled in size over the course of 7 days (with concurrent increase in bioluminescence), whereupon it became inflamed and ulcerated and the animal was sacrificed. Similar increases in size were observed in CTL treated mice with no ulceration, however tumour development was equally rapid with no apparent regression or plateau of tumour growth as a result of CTL activity.

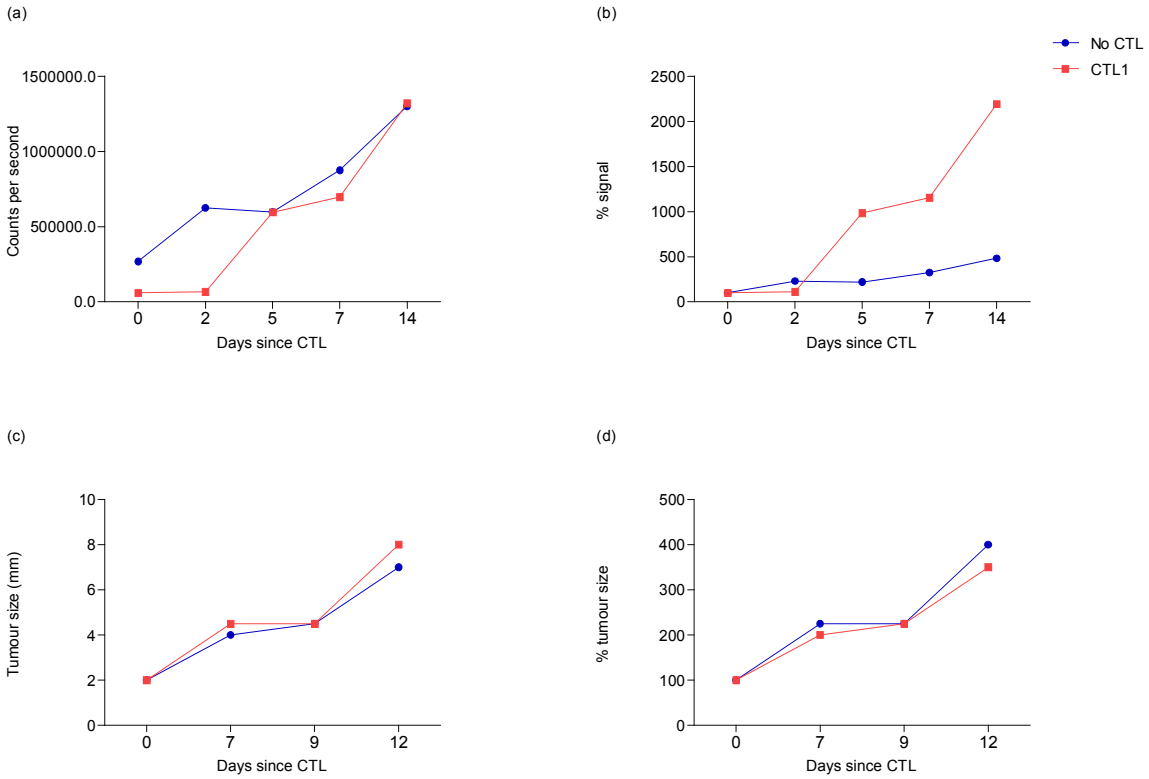


Figure 60: Bioluminescence and size monitoring of EBV-CTL treated and untreated tumours established from donor F LCL. (a) Absolute light emission from ROI. (b) Light emission relative to signal on day of CTL injection. (c) Tumour size measured in mm. (d) Tumour size relative to size on day of CTL injection.

Preliminary data from donor F (Figure 60) shows that under these conditions, administration of autologous EBV-CTLs to mice bearing LCL tumours also did not lead to tumour regression or delay in progression. One animal was treated with CTLs and one untreated with no resulting differences in tumour progression.

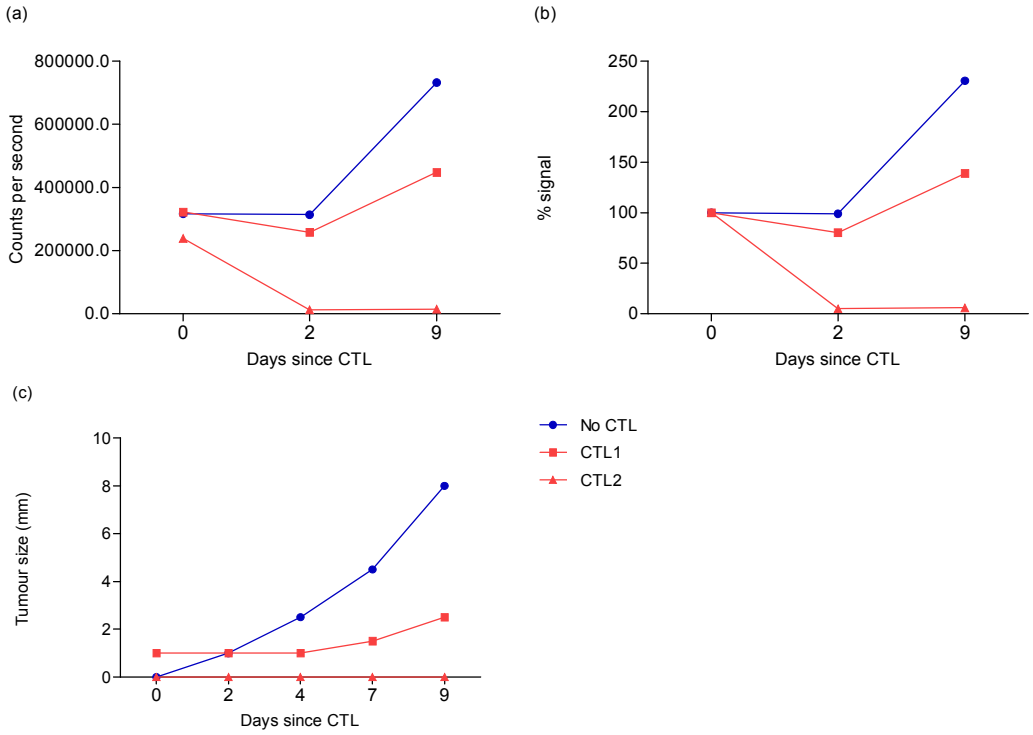


Figure 61: Bioluminescence and size monitoring of EBV-CTL treated and untreated tumours established from donor D LCL. CTLs were injected into treated mice (red lines) on day 0. (a) Absolute light emission from ROI. (b) Light emission as percent of that on day of CTL injection. (c) Tumour size measured in mm.

However, results obtained from donor D showed a probable effect of CTL infusion, with a rapid increase in growth of tumour in the non-treated mouse, and concomitant increase in FLuc signal detected on imaging. In contrast, one of the mice treated with EBV-CTLs (CTL2) appeared to experience regression of the LCL tumour and the other (CTL1) had slower disease progression compared to the untreated control. On the day of CTL infusion into animals CTL1 and CTL2, the untreated animal and CTL2 from this donor showed clear signal from the injection site from imaging data, however the palpable tumour was negligible. As the tumours could not be measured on this day, adjustment of the subsequent size measurements to baseline size as performed for FLuc measurements was not possible. At no point did animal CTL2 develop a tumour large enough to measure with callipers, and the FLuc signal dropped to background, whereas the untreated animal developed a large tumour expressing

high levels of FLuc (see Figure 61 and Figure 62). These preliminary results suggested that the i.v. administration of donor D EBV-CTLs to mice bearing s.c. donor D LCL tumours may result in delayed progression, or regression of the tumour.

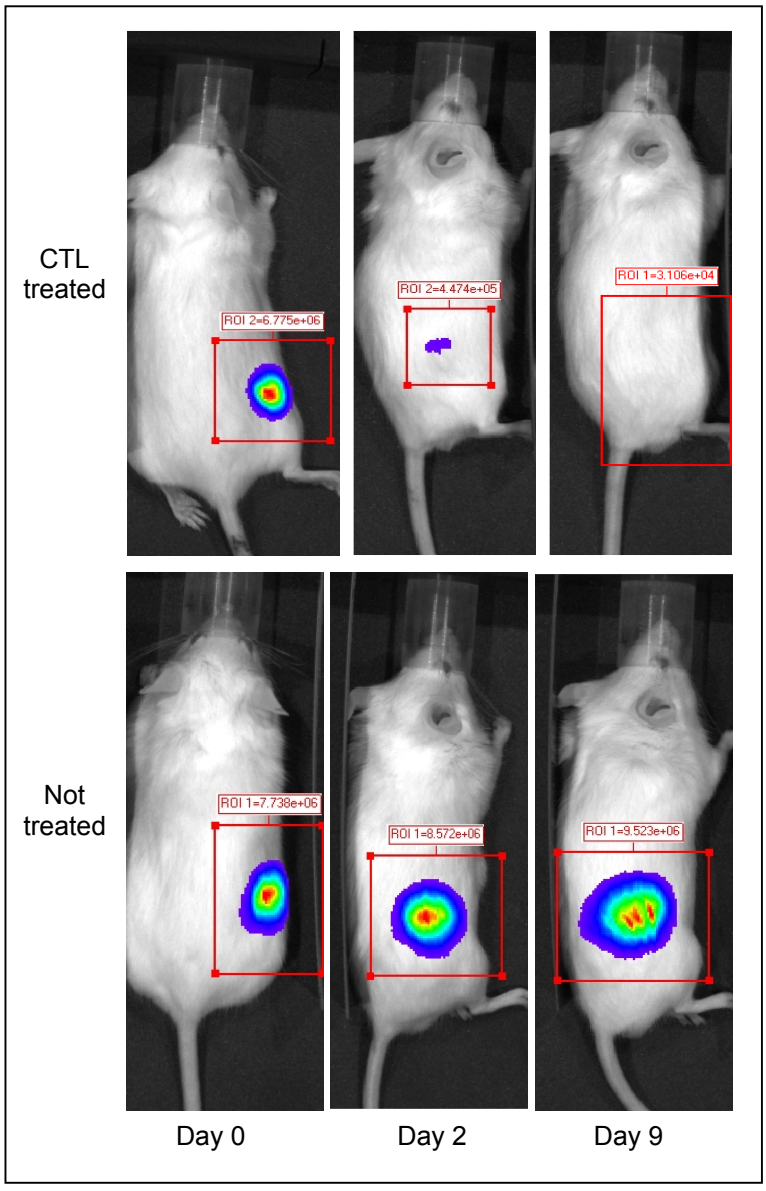


Figure 62: Regression of donor D LCL tumour in CTL treated but not untreated mouse. CTLs were administered when LCL tumour was detectable, mice were monitored by bioluminescence and tumour measurement. Apparent regression of the tumour was observed with both methods in the treated animal. In contrast, the tumour in the untreated animal grows rapidly.

In addition, it is important to note that the FLuc signal obtained from imaging correlates with the measured size of tumours. Bioluminescence is considerably more sensitive than physical measurement, being capable of detecting signal from transduced LCL prior to the presence of a palpable tumour. Furthermore, there is a sizable contribution of handler experience in measurement of tumours with callipers, compounded by the observation that tumours are more readily detectable by palpation when the animal is anaesthetised compared to a conscious animal. Therefore, bioluminescence imaging with the IVIS system was subsequently used to monitor tumour growth.

5.5.2 Identification of human T cell infiltrate in LCL tumour

Tumours from the untreated animal and CTL1 from donor D were excised and analysed histopathologically. The site of injection from animal CTL2 was examined, however minimal residual tumour was identified. As shown in Figure 63, heavy infiltration of CD3 positive human T cells was detected in CTL treated but not untreated tumours. Tumours from other cohorts not experiencing an effect of EBV-CTL administration showed either no CD3 infiltrate or only sparse CD3 positive cells persisting 12-16 days after administration (data not shown).

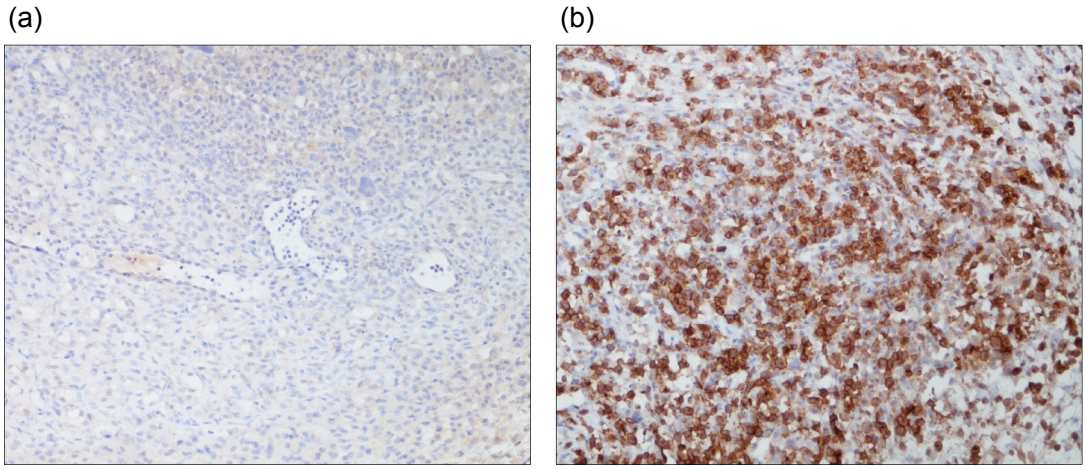


Figure 63: Donor D LCL tumour section stained for human CD3 expression shows heavy T cell infiltrate. (a) Tumour excised from untreated control negative for CD3 staining. (b) Tumour from CTL treated mouse CTL1 shows heavy infiltrate of human T cells 9 days post injection. 100x magnification.

These preliminary data indicated that while there was significant donor to donor variation in the efficacy of EBV-CTLs in treating/preventing LCL tumours in 3KO mice, our approach could be tested in donor D. With this donor, we observed regression or delayed progression of LCL tumours in treated compared to untreated animals and demonstrated infiltration of human CD3 positive cells into the tumour by histopathological analysis. We therefore designed subsequent experiments to confirm these data, and to investigate the effect of infusing cells prophylactically compared to treating an established tumour.

5.5.3 Comparison of EBV-CTL prophylaxis and treatment

In order to examine the effect of CTL administration as either prophylaxis or treatment for EBV-PTLD, $5x10^8$ EBV-CTLs were generated from donor D, and cryopreserved following stimulation four for administration when required.

Nine 3KO mice were inoculated s.c. with 10^7 donor D LCL as previously. Three mice (control cohort) received 2500U IL-2 i.p. three times weekly but no CTLs. A further three mice (prophylaxis cohort) were given 10^7 viable EBV-CTLs i.v. on the same day as LCLs. All animals were imaged on the day of LCL injection to establish a baseline for signal from 10^7 LCL. Subsequently, animals were imaged twice weekly until signal from the site of injection increased on two consecutive occasions, indicating establishment and growth of the LCL tumour. The sensitivity of bioluminescence imaging is illustrated by the clear signal obtained on day 0 of this experiment, immediately following LCL and D-luciferin administration. Interestingly, FLuc signal from the site of LCL injection dropped in all mice treated at 4 days post injection, before returning to approximately baseline values on day 8 and increasing by day 11 (see Figure 64). It is likely that this drop represents the period of LCL engraftment prior to vascularisation and establishment of the tumour. Finally, a third cohort (treatment cohort) received EBV-CTLs i.v. on day 11 post LCL administration. Tumour size was then monitored three times weekly and imaged once weekly.

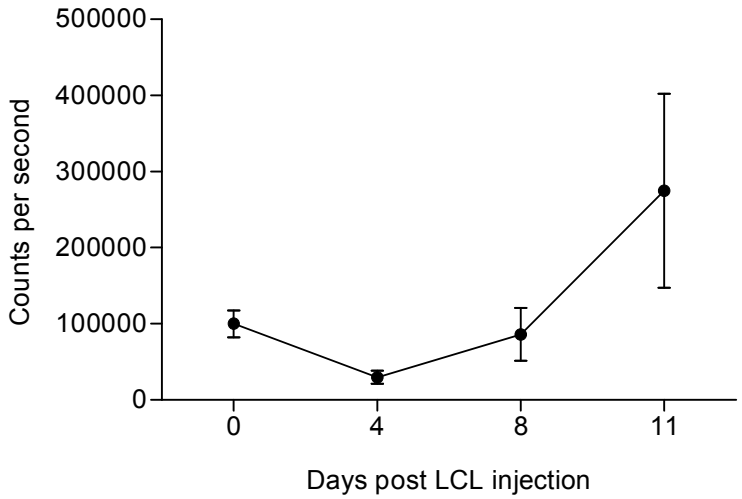


Figure 64: Luciferase signal from the site of LCL injection. Tumour growth was monitored bi-weekly for 11 days. Signal dropped from all animals at day 4, but subsequently rose at days 8 and 11 (*n=9*).

Animals were imaged every 5-7 days following administration of CTLs to the treatment cohort to monitor tumour progression. As shown in Figure 65, despite the previous results obtained from this model using this donor, infusion of EBV-CTLs had no effect on tumour growth in either the prophylactic or the treatment cohorts in this experiment.

Tumour samples were once more analysed for T cell infiltrate however, in contrast to previous data, sparse scattered T cell clusters only were identified in the treated mice. In addition, peripheral blood, spleen and liver samples were analysed by flow cytometry for the presence of human T cells, with no CD3 positive cells identified at any of these sites (data not shown), possibly suggesting that adoptively transferred CTLs do not survive long term in this mouse model, even with exogenous IL-2 support.

Further optimisation of this PTLD treatment model is required to determine the parameters necessary to induce reliable tumour regression. The possible approaches to this optimisation will be discussed in section 5.7.1.

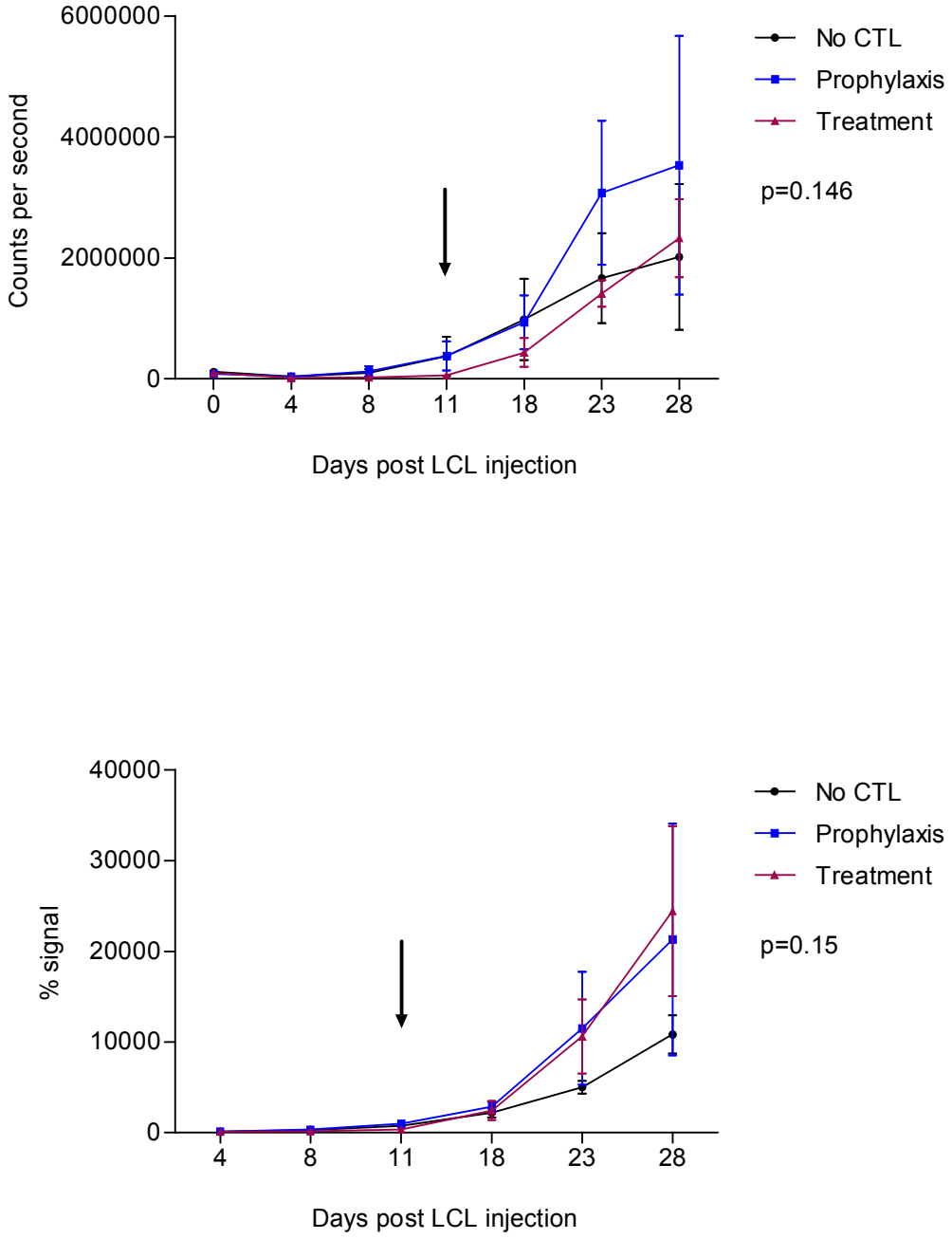


Figure 65: Equivalent tumour progression in untreated, prophylactic CTL or treatment CTL groups. (a) Absolute counts per second from the injection site. (b) FLuc signal normalised to lowest value for each animal. Mean and SEM shown, n=3 per group. Administration of CTLs in treatment group indicated by arrow. Statistical analysis using two-way ANOVA.

5.6 Effect of calcineurin inhibitors on transduced and untransduced CTLs *in vivo*

5.6.1 Administration of CN inhibitors

Prior to assessing the effect of CN inhibitors on the function of EBV-CTLs *in vivo*, the appropriate route and dosage must be determined. Various routes of administration including gavage have been employed by investigators in the literature. While this approach would not be possible for the duration of our experiments, these studies demonstrate that oral absorption of FK506 is possible and we initially investigated the possibility of administering this drug via the drinking water. This would allow long term dosage of animals without the need for daily i.p. injections which would introduce both practical and licencing issues. Although FK506 is not soluble in water, we utilised an intravenous liquid preparation which was diluted to the appropriate concentration and added to the sterile drinking water. The water bottle was agitated daily to ensure homogenous delivery of FK506 in water.

To ascertain the correct concentration of FK506 to be added, an approximation of the volume of water consumed per day per mouse was necessary. Therefore the water bottles of one cage of two male strain 3KO mice and one singly caged female were weighed and monitored over two days. This determined that each mouse consumed approximately 8.5ml of water per day. In addition, the mice were weighed and were found to weigh an average of 20g per animal. The normal dose of FK506 administered to paediatric transplant patients is 300μg/kg/day, therefore to achieve similar dosing each mouse should consume 6μg/day. To account for bottle leakage, it was assumed that each mouse drank 6ml water per day and FK506 was added at a concentration of 1μg/ml. There are several potential problems with this route of administration and dosage assumptions. Firstly, mice caged in groups are likely to consume differing volumes of water and will therefore receive different doses of CN inhibitor. Secondly, although FK506 has been shown to be stable at room temperature and up to 30^{o} for several days in blood samples (Alak and Lizak 1996), there is the possibility of drug breakdown. Finally, and most improtantly, the pharmacokinetics and metabolism of

FK506 in mice is not well established and therefore to obtain an adequate circulatory concentration, extrapolation from the human paediatric dose may not be sufficient.

Therefore, following three days of FK506 administration and upon observation of no ill effects, the animals were sacrificed and cardiac puncture was performed to obtain sufficient sample for measurement of blood FK506 concentration (see Table 14). These results showed that FK506 concentration was undetectable (<2ng/ml) in all three mice. Therefore the experiment was repeated in two animals with a four fold higher concentration of FK506 in the drinking water, to administer 24μg/day per mouse, equivalent to a human dose of 1.2mg/kg/day. In addition, the sample was collected from one animal in the morning and one in the evening, representing a peak and trough concentration respectively as mice consume more water during the night compared to the day. However, FK506 concentration in these samples was again undetectable. A repeat of the experiment with administration of a ten-fold increased dose also produced undetectable blood concentration of FK506, therefore this approach was not pursued (Table 14).

Table 14: Administration of FK506 intravenous preparation orally in drinking water does not result in detectable concentrations in circulation.

Sample	Dosage (x paediatric dose)	Measured blood concentration (ng/ml)
1	6μg/day (1x)	<2
2	6μg/day (1x)	<2
3	6μg/day (1x)	<2
4	24μg/day (4x)	<2
5	24μg/day (4x)	<2
6	60μg/day (10x)	<2
7	60μg/day (10x)	<2
8	60μg/day (10x)	<2

Finally, dosing mice via the oral route using drinking water was tested using both FK506 and CsA oral syrup preparations. We once again administered 10 fold increased dose of FK506, and CsA to three mice, and measured blood concentrations. As shown in Table 15, again FK506 was not present in the blood at a therapeutic concentration. In contrast however, CsA was absorbed when administered using this route and dosage and levels within and above the human therapeutic range were achieved. These results were obtained from three littermates caged together, and our data highlights that mice achieve varying plasma concentrations even when drinking from the same water bottle. This may reflect either differences in the dose received or in drug metabolism between animals.

Table 15: Successful administration of CsA but not FK506 oral syrup via drinking water.

Sample	Dosage (x paediatric dose)	Measured blood concentration (ng/ml)
1	60μg/day FK506 (10x)	<2
2	60μg/day FK506 (10x)	<2
3	60μg/day FK506 (10x)	<2
4	CsA 1.2mg/day (10x)	288
5	CsA 1.2mg/day (10x)	564
6	CsA 1.2mg/day (10x)	730

5.7 Conclusions

These results confirm that reliable establishment of human LCL tumours in 3KO mice is possible for the majority of donors. LCL establish tumours and become palpable within one to three weeks of injection, depending on the donor, with progression to the size limit of 10mm stipulated on the project licence within approximately two to five weeks.

We have established and optimised bioluminescent imaging of FLuc transduced LCL tumours in 3KO mice, determining the appropriate parameters and protocol to use for reliable results. Signal emitted from tumours has been correlated with tumour size as measured by callipers, this was confirmed to be a sensitive method of detecting LCL immediately following injection, and a reliable indicator of tumour growth.

Having established the *in vivo* model of PTLD development and a reliable non-invasive monitoring technique, we next addressed the efficacy of treatment for EBV-LCL tumours with autologous EBV-CTLs. Preliminary experiments identified mice from one of three donors appearing to respond to CTL treatment with delayed tumour progression or indeed regression following infusion of CTLs. This variability between donors could be due to a number of factors, including the observation that in the two donors not responding to EBV-CTL treatment, the LCLs proliferate more rapidly in *in vitro* culture than those of the successful donor. *In vivo* this translated in one donor to very rapid tumour growth and ulceration at the tumour site after only 7 days. To control rapidly proliferating LCLs such as these, it is possible that a higher CTL dose would be required for these donors. However, for the donor responding to CTL infusion, we identified heavy T cell infiltrate in the remaining tumour of one animal, and the absence of significant tumour tissue at the injection site for the animal in which regression was observed. These results were not replicated in a secondary series of experiments from the same donor aiming to compare prophylaxis with treatment of LCL tumours. Several possibilities will be explored to optimise this model and improve the reliability of responses to CTL infusion.

5.7.1 Further optimisation of EBV-PTLD model

To improve EBV-CTL treatment for LCL tumours in 3KO mice, further optimisation is required. Preliminary data in this study gave promising results, therefore successful establishment of this model is likely.

Initially we will focus on practical issues possibly affecting the function of EBV-CTLs following infusion, such as minimising the delay between preparation of the cells for injection and administration of these cells. Currently these two procedures occur at different sites within our institution therefore some time elapses which may have a detrimental impact on CTL function. In further studies, CTLs will be thawed and prepared at the site of animal maintenance, therefore reducing this delay.

Secondly, our successful pilot studies utilised EBV-CTLs that were cryopreserved following two stimulations, whereas in the unsuccessful follow up experiments, EBV-CTLs received four stimulations prior to cryopreservation. Gattinoni *et al* demonstrated that CTLs with a shorter *in vitro* culture period perform more effectively *in vivo* as a result of improved homing to the tumour site, therefore it is possible that this could impact the function of CTLs (Gattinoni*, et al* 2005). However, CTLs used in the study by Lacerda *et al* had received either three or four stimulations prior to administration and resulted in dramatic tumour regression (Lacerda*, et al* 1996).

Next the distribution of the infused CTLs will be examined to determine their fate following administration; despite the absence of human T cells from the liver and spleen in our final experiment, this analysis was performed 17 days post T cell injection and CTLs would be unlikely to persist in these sites in the absence of antigen stimulation. EBV-CTLs will therefore be transduced with FLuc and these effectors administered to mice bearing an LCL tumour, with bioluminescent monitoring to track their distribution immediately post injection and in the days following treatment. This could identify whether CTLs home to the tumour site but are not sufficiently functional to induce tumour regression, or whether they do not localise to the tumour but are cleared from the animal by the liver or spleen before arrival and are unable to mediate anti-tumour effect.

In addition to these factors, providing the optimal cytokine environment for CTL survival and function will also be examined. CTLs are currently thawed one day prior to injection and rested overnight in 40U/ml IL-2 before administration. Optimisation of the cytokine cocktail during this rest period may improve the function of CTLs following infusion. In addition, 2500U IL-2 is administered to the animals i.p. three times weekly following CTL infusion, however it is possible that this dose is insufficient for CTL effect and persistence, or that administration of additional or alternative cytokines such as IL-15 would improve the environment (Mueller*, et al* 2008, Quintarelli*, et al* 2007, Rowley*, et al* 2009). Another approach to provide a constant source of cytokines, rather than the peaks and troughs that likely result from multiple injections, would be transduction of the LCL tumour cells with cytokine genes. This would provide constant expression of IL-2/IL-15 at the site of the tumour, thus enhancing CTL activity to augment the anti-tumour response *in vivo*. Potentially, LCL could also be transduced with other immunostimulatory molecules such as the T cell chemokine lymphotactin to enhance T cell trafficking to the site of the LCL tumour (Dilloo*, et al* 1996).

5.7.2 Optimisation of CN inhibitor administration

To examine the effect of FK506 on transduced compared to untransduced EBV-CTLs in the treatment of LCL tumours *in vivo*, it is critical to address the issue of correct dosing of the animals. Several routes of administration have been employed in previously published studies, however resulting blood concentrations have not been established. We attempted oral dosing of animals using an i.v. preparation of FK506 administered in drinking water and observed undetectable blood concentrations with up to ten fold increased dose compared to paediatric transplant patients. This result was replicated using an oral syrup preparation of FK506 at the same dose, however administration of an oral preparation of CsA using this route resulted in considerable, although variable, circulatory concentrations.

These results highlight the difficulty in accurately dosing mice with CN inhibitors which are poorly water soluble and metabolised rapidly, requiring administration of

high doses to achieve therapeutic blood concentrations. Potentially however, we could use orally administered CsA after further experiments to determine that we are able to consistently achieve drug levels within or above the therapeutic range without toxicity. While the circulating CsA levels achieved appear variable between mice, given that CTLs transduced with CNb30 are able to function at supratherapeutic levels of CsA *in vitro*, this variability may not be critical for our subsequent experiments as long as therapeutic drug levels are consistently obtained.

Other routes of administration described in the literature include i.p. and i.v. injection, both of which present practical and licencing difficulties. A further approach currently under investigation is the use of osmotic pumps to administer a continuous s.c infusion of drug over a period of up to four weeks. This will allow high dosage and the continuous nature should partly address the issue of rapid metabolism in our murine model.

In conclusion, establishment of an appropriate system for examining the efficacy of transduced EBV-CTLs in the presence of CN inhibitors *in vivo* is underway. Establishment and monitoring of such tumours is possible, however several factors remain to be finalised regarding CTL treatment and CN inhibitor administration, and optimisation of these aspects is ongoing.

5.8 General conclusions

- Successful establishment of murine model of PTLD development for *in vivo* studies.
- Bioluminescence imaging of LCL tumour growth and regression is possible and has been optimised.
- Treatment of murine PTLD with EBV-CTLs has been variably successful and requires further optimisation.
- Administration of FK506 via the oral route is not possible using the methods tested here. Administration of CsA is possible but results in variable circulating drug levels.

Chapter six

Discussion

6.0 Discussion

6.1 Study conclusions

The development of EBV-driven PTLD following stem cell or solid organ transplantation is a serious complication of these procedures. Occurring in between 1 and 30% of patients, PTLD can be an aggressive disease and carries a mortality rate of up to 50% at 5 years (Everly*, et al* 2007, Opelz and Dohler 2004, Taylor*, et al* 2005, Webber*, et al* 2006). An increasing number of patients undergo transplantation, with improved life expectancy due to a combination of advances in HLA matching, conditioning regimes and immunosuppressive therapy. Therefore, a larger number of patients are at risk of developing post transplant complications such as PTLD. The poor prognosis of this disease has driven the research effort to improve prevention strategies and investigate novel treatments, many of which focus on the use of immunotherapy.

Adoptive immunotherapy for EBV-driven post transplant lymphoma has proven both safe and effective, with administration of EBV-CTLs to over 200 patients to date (Merlo*, et al* 2008). In the setting of stem cell transplantation, this approach has been shown to both prevent the development of PTLD when administered prophylactically and induce regression of established tumours (Rooney*, et al* 1998). However, while some clinical responses have been observed following the application of this approach following solid organ transplant, both the magnitude and persistence of these have been limited (Comoli*, et al* 2002, Comoli*, et al* 2005, Savoldo*, et al* 2006, Sherritt*, et al* 2003). Transient reductions in EBV viral load or increases in EBV-CTL precursor frequency are documented, along with reduction in tumour burden, but the lack of CTL persistence following transfer along with relapses observed after this treatment raise questions regarding the persistence of such cells in the SOT recipient (Khanna*, et al* 1999). While a recent phase II study of partially HLA-matched third party CTLs showed good response rates of 52% at 6 months (Haque*, et al* 2007), the efficacy of adoptive immunotherapy with EBV-CTLs after SOT has generally been less impressive than in the SCT setting. It is likely that the presence of ongoing

immunosuppressive therapy may serve to limit the survival and efficacy of the infused cells in this environment (Savoldo, *et al* 2006).

To address this issue, we have generated EBV-specific CTL lines that retain functionality in the presence of CN inhibitors. This approach may improve the efficacy of adoptive immunotherapy in this setting, allowing continuation of the immunosuppressive regimen to protect from graft rejection, whilst enabling efficient function of the therapeutic product to combat EBV-induced B cell proliferation. To this end, we generated a panel of 54 CN mutants designed to resist binding by either or both of the CN inhibitors FK506 and CsA. We hypothesised that CN mutants able to resist this binding would retain their functionality in the presence of CN inhibitors and therefore confer resistance to these drugs. CN mutants were generated based on previously identified resistance-conferring mutations in conjunction with structural data (Cardenas, *et al* 1995, Fox, *et al* 2001, Griffith, *et al* 1995, Kawamura and Su 1995, Ke and Huai 2003, Milan, *et al* 1994). These mutations were designed to disrupt the binding of CN by CN inhibitors, whilst preserving both the interaction between CNa and CNb and the phosphatase activity of the enzyme. Extensive screening of all mutants using a Dual Luciferase Assay in 293T cells enabled identification of 17 mutants that retained some phosphatase activity in the presence of either FK506 or CsA, therefore these mutants were pursued in further experiments. Generation of retroviral supernatants containing the 17 selected CN mutants and transduction of Jurkat T cells allowed assessment of the effect of these mutants on IL-2 secretion by T cells in the presence of CN inhibitors. These results confirmed that the introduction of mutants CNa12, CNa22 or CNb30 into Jurkat cells conferred the highest resistance to suppression with CN inhibitors. Indeed, transduction with these three CN mutants enabled IL-2 secretion not only at therapeutic levels of FK506/CsA but also at supratherapeutic levels up to four fold higher than this. CNa12 was selected to confer resistance to FK506, CNa22 for resistance to CsA, and CNb30 conferred resistance to both CN inhibitors (chapter three).

As described above, the molecular basis for the ability to resist binding by CN inhibitors is due to alterations in CN which prevent binding by FK506/CsA (see table 4). CNa12 contained the novel combination of two mutations T351E and L354A, first

identified by Kawamura (Kawamura and Su 1995). These substitutions were predicted to disrupt binding between CNa and the charged surface residues H87-P88 of FKBP12 but should not affect CsA/CyPA binding, resulting in FK506 but not CsA resistance. CNa22 contains the mutations V314R, which directly disrupts CsA binding and Y341F, which may prevent FKBP12/CyPA binding by inhibition of close contact with the body of CNa. CNb30 contains both the point mutation L124T, decreasing the size and charge of the side group, and the insertion K125-LA in the latch region, which is involved in binding of CNb to CNa and also in binding of FKBP12/CyPA to the CN heterodimer. Insertions at this point in the latch region are likely to disrupt the overall structure of the loop, resulting in a protrusion outwards and away from contact with the immunophilin (Fox*, et al* 2001).

The three CN mutants identified as conferring resistance during cell line assays were subsequently codon optimised and introduced into primary EBV-CTL lines. In the absence of CN inhibitors, proliferation, cytokine secretion, phenotype, cytotoxicity and dependence upon antigen stimulation were monitored for four weeks *in vitro* and were established to be unaffected by transduction with CN mutants under normal culture conditions. Therefore neither the transduction procedure nor expression of CN mutants has a detrimental effect on EBV-CTL function.

Addition of CN inhibitors to culture conditions resulted in suppression of UT EBV-CTLs, with a marked reduction in proliferation and cytokine secretion. In contrast, CN mutant transduced EBV-CTLs were able to expand normally in the presence of therapeutic levels of FK506/CsA, with comparable growth to that seen by the same CTL lines in the absence of CN inhibitors (53-106%) as measured by the increase in cell numbers. This ability to proliferate was confirmed by ^{3}H-Thymidine uptake assays. In addition, proliferation of CNb30 transduced EBV-CTLs was examined in the presence of supratherapeutic levels both of FK506 and CsA and found to be normal. CTL functionality in the presence of CN inhibitors was examined by measurement of IFN-γ secretion in response to stimulation with autologous LCL. Normal levels of IFN-γ secretion were observed in the presence of either FK506 (CNa12, CNb30) or CsA (CNa22, CNb30) (chapter four). In addition we have shown that the cytotoxicity of EBV-CTL lines is unaffected either by growth in CN inhibitors

and inclusion of these drugs in the cytotoxicity assay itself, or by transduction with CN mutants. Thus, as determined by the endpoints of proliferation, IFN-γ secretion and cytotoxicity, transduction with these CN mutants rendered EBV-CTL lines resistant to suppression with CN inhibitors. This approach may therefore enable adoptively transferred CTLs to function in SOT patients at risk of PTLD, or in whom PTLD has developed, without the need to reduce immunosuppression with CN inhibitors and the concurrent risk of graft rejection.

The observation that transduced EBV-CTL lines are able to proliferate at CN inhibitor doses four-fold above the therapeutic range is likely to be reflective of the mechanism of action of the resistance conferred by CN mutants. As the CN inhibitor: immunophilin complex is not bound by the CN mutant, the dose of FK506 or CsA administered is irrelevant, allowing resistance even at high doses. This finding may increase the potential applications of CN inhibitor resistant cells, allowing administration in the immediate post-transplant period when doses of CN inhibitors are high. In addition, raising the dose of CN inhibitors without having a detrimental impact on CTL function may enable the withdrawal of other immunosuppressive drugs such as MMF to allow effective immunotherapy during this time.

The generation of mutants capable of conferring resistance to either or both CN inhibitors allows a choice of approach with potential therapeutic advantages to either. CTLs resistant to one CN inhibitor could be suppressed using the alternative CN inhibitor if necessary. For example, inflammatory reactions at the site of the tumour have been documented following infusion of EBV-CTLs when used as treatment for established PTLD (Rooney*, et al* 1998). If such an adverse event occurred, CTLs resistant to only one CN inhibitor could be suppressed by administration of the other. Since CTLs would not be deleted using this approach, EBV-specific memory would be retained long term. Conversely, EBV-CTLs resistant to both FK506 and CsA would allow a choice of therapeutic agent. Patients experiencing toxicity as a result of CsA use could be switched to FK506 without suppression of infused EBV-specific cells. An additional benefit of mutant CNb30 is a result of the small size of the CNb gene. At approximately 500bp, the size of CNb is well below the packaging limit of

integrating vectors, therefore allowing co-expression with other useful transgenes and extending the applicability of this approach.

In summary, CN mutants capable of conferring resistance to suppression with CN inhibitors have been generated and examined in both cell line screening assays and primary EBV-CTL lines. This approach may improve the efficacy of immunotherapy for EBV-driven PTLD arising post SOT.

6.2 Safety

The major safety concerns regarding the use of CN transduced EBV-CTLs are the possibility of hyper-activation induced by increased cellular CN, the effects of the transduction itself, and the potential for generating allo-reactive CTLs that are resistant to CN inhibitors. This study addressed these important issues, confirming both that hyperactivity is not observed in transduced EBV-CTLs, that transduction alone does not affect EBV-CTL function and that alloreactivity is not increased by CN transduction.

6.2.1 Risks relating to transgene expression

During CN mutant screening in Jurkat cells, it was noted that transduction with either wt or mutant CNa genes resulted in increased secretion of IL-2 from Jurkat cells upon stimulation with mitogens. The observed hyper-activity of Jurkat cells following transduction with CNa is likely due to the presence of additional CN in the cell amplifying the activation signal and leading to increased IL-2 production. This finding indicates that CN activity is the limiting step in activation of IL-2 secretion by the Jurkat T cell line. Interestingly, hyper-activation is several fold more pronounced following transduction with CNa genes than CNb. This result, in combination with Western blot data demonstrating increased levels of CNb following transduction of cells with CNa, supports the suggestion that CNa is the limiting component of the CN heterodimer and that binding by CNa stabilises CNb, which may otherwise be

degraded (Milan*, et al* 1994) (figure 29). Importantly for future clinical studies, this suggests that transduction with CNb mutants may be capable of conferring CN inhibitor resistance whilst not raising cellular CN levels, therefore not resulting in hyper-activation or otherwise affecting cellular function. The generation of a cellular therapeutic product with the potential for over-activity or an increased level of functionality is clearly undesirable, as this could potentially result in inappropriate proliferation therefore generation of transduced EBV-CTLs that do not display any gain-of-function is of utmost importance. Although the development of neoplasia is unlikely as we are transducing differentiated T cells, inappropriate T cell activation could potentially result in alternative side effects such as the induction a cytokine storm with consequent inflammation. While increased activation of Jurkats was observed following PMA/ionomycin treatment, no detectable IL-2 secretion occurred in the absence of stimulation either from UT or CN transduced cells. This confirms that increased CN appears able to amplify an activation signal, but is not capable of initiating such activation itself. Additionally, increased wtCN alone did not result in resistance to CN inhibitors; approximately 300% increased levels of IL-2 secretion was observed from wtCNa transduced cells upon stimulation, however this excess IL-2 production remained sensitive to suppression by FK506/CsA, and was abrogated by their addition.

Despite the evident hyper-activation in Jurkat cells transduced with CNa, no such effect was observed in EBV-CTLs despite close monitoring for four weeks *in vitro*. Transduced EBV-CTLs were examined in the absence of CN inhibitors by assessment of cell expansion, proliferation by ^{3}H-thymidine uptake assays, and secretion of IFN-γ. These data confirmed that transduction with CN mutants does not result in increased proliferation or IFN-γ secretion by EBV-CTLs even with CNa22, which resulted in the highest level of hyper-activation in Jurkat cells. This difference between Jurkat cells and primary EBV-CTLs may be as a result of the strength of the activation signal provided, which is stronger in the former (PMA/ionomycin) and more physiological in the latter (LCL). Alternatively, as discussed in chapter 4, it is possible that EBV-CTLs overexpressing CN mutants such as CNa22 undergo increased rates of activation induced cell death. The observation of a gradual drop in the proportion of CNa22 transduced CTLs in the absence of CN inhibitors is consistent with this possibility. In

summary, our data indicate that transduction of EBV-CTLs with CN mutants appears not to result in hyper-activity.

6.2.2 Risks relating to retroviral gene transfer

The use of viruses in gene transfer carries the risk of inappropriate activation of surrounding genes at the site of integration into the genome. The effects of such insertional mutagenesis have been highlighted by the severe adverse events observed in two gene therapy trials in recent years where CD34-selected stem cells have been transduced with retroviral vectors (Hacein-Bey-Abina*, et al* 2008, Hacein-Bey-Abina*, et al* 2003, Howe*, et al* 2008). These events have driven investigations into developing safer viral vectors to reduce the likelihood of insertional mutagenesis. These include the development of the self-inactivating vector and the use of lentiviruses rather than retroviruses to improve the safety profile of such constructs. However, aside from the fact that these vectors have not been utilised in any clinical trial to date, such vectors carry several disadvantages such as the production of lower titre supernatants and the inability to generate stable producer lines for lentiviral vectors, and the lower titre of SIN retroviruses. However, in our approach, we are transducing mature T cells rather than stem cells, which (as discussed in section 1.5.3) reduces the potential for leukaemagenesis. Indeed, transduction of mature T cells with retroviral vectors has not been associated with any severe adverse events despite treatment of over 100 patients to date with transduced T cells (Blaese*, et al* 1995, Ciceri*, et al* 2007, Ciceri*, et al* 2009, Hale*, et al* 2008, Tiberghien*, et al* 2001), including 40 patients treated with retrovirally transduced EBV-CTLs. Therefore in our studies we have used the retroviral vector SFG for transduction of EBV-CTLs, from which it is possible to generate high titre, stable producer lines to produce viral supernatant on a large scale, resulting in good CTL transduction efficiency. In view of the differentiated nature of EBV-CTLs and the existing data from the clinical studies above, we believe that the potential benefit from using a conventional retroviral vector outweighs the theoretical risk of leukaemagenesis.

In conclusion, investigation of CN mutant transduced EBV-CTLs in the presence or absence of CN inhibitors demonstrates that transduction with CN mutants does not result in hyper-activity or increased allo-reactivity and that such cells are not adversely affected by the transduction procedure. Therefore the favourable safety record for immunotherapy with EBV-CTLs is likely to be maintained in CN transduced cell lines. To further increase the safety profile of CN transduced EBV-CTLs, inclusion of a suicide gene such as HSV-TK (Ciceri*, et al* 2009) or iCaspase9 (Straathof*, et al* 2005) in the CNb30 vector is possible. This would allow elimination of transduced CTLs should an adverse event attributable to the administration of these cells occur. Alternatively, if CNa12 or CNa22 were used, CsA or FK506 could be used to suppress transduced cells.

6.2.3 Risks relating to CTL use

The major risks associated with adoptive immunotherapy with EBV-CTLs are alloreactivity and inflammatory reactions. Due to the potential presence of alloreactive cells in the original PBMC sample, EBV-CTL lines prepared for clinical use are cultured *ex vivo* for several weeks prior to reinfusion (Comoli*, et al* 2005, Khanna*, et al* 1999, Rooney*, et al* 1995, Wilkie*, et al* 2004). Multiple stimulation of CTLs with autologous LCL during the *ex vivo* procedure has the dual effect of both enhancing the proportion of EBV-specific cells and reducing allo-reactivity, thus ensuring the specificity of the cell line and minimising the potential for initiation of either GVHD in the SCT setting or graft rejection in SOT patients. During this process, allogeneic cells undergo apoptosis due to lack of stimulation, as highlighted by the reduction in allo-specific cytotoxicity concomitant with increased number of stimulations *in vitro* (Wilkie*, et al* 2004). This approach appears to effectively deplete alloreactivity, as evidenced by the fact that administration of such cells to over 200 patients has not been associated with increased GVHD or graft rejection (Heslop*, et al* 2009, Merlo*, et al* 2008). Importantly, this study demonstrated no increase in cytotoxicity towards allogeneic targets in CTL lines as a result of CN transduction. Therefore, we believe that the risk of generating allo-reactive, CN inhibitor resistant CTLs as a result of this procedure is minimal.

6.3 Additional obstacles to adoptive immunotherapy

In addition to the ongoing requirement for immunosuppressive therapy, several other potential obstacles may limit the efficacy of adoptively transferred CN inhibitor resistant EBV-CTLs after SOT. These include the use of combination immunosuppressive regimens, the lack of immunological space, the dependence of the infused cells on stimulation with specific antigen, and the possibility of an immune response to the transgene, along with practical considerations such as time, facilities and resources.

6.3.1 Suppression of CTLs by other immunosuppressive agents

Although CN inhibitors form the cornerstone of many immunosuppressive regimens, combination therapy with other agents, particularly Mycophenolate Mofetil (MMF) and/or steroids, is usually employed (Caillard*, et al* 2005, Knight*, et al* 2009). Therefore, the function of EBV-CTLs that are resistant to FK506/CsA may still be impaired by these additional agents when infused post transplant. However, first line treatment for EBV-PTLD includes reduction of immunosuppression to as low a level as possible, which in many centres involves complete withdrawal of other agents and reduction to the lowest allowable dose of CN inhibitors (Everly*, et al* 2007, Svoboda*, et al* 2006, Tsai*, et al* 2001). In general, withdrawal of MMF is well tolerated and where rejection has been seen this is most frequently due to over-aggressive reductions in CN inhibitors. Withdrawal of additional agents would allow the function of CN inhibitor resistant EBV-CTLs. Indeed, since transduced EBV-CTLs function at supratherapeutic levels of CN inhibitors, potentially the dose of CsA/FK506 could be increased to compensate for withdrawal of other agents. Alternatively, where EBV-CTLs are infused prophylactically and maintenance of standard immunosuppression is desirable, it may be possible to generate CTLs that are resistant to both CN inhibitors conferred by CNb30 and MMF conferred by mIMPDH2. Transduction with mIMPDH2 has been shown to render T cells resistant to suppression with MMF in terms of both proliferation and cytokine secretion (Yam*, et al* 2006). Therefore our laboratory is currently investigating combining these two approaches to produce

FK506/CsA/MMF resistant cells. Because of the small size of CNb30 (500bp) and IMPDH2 (1.5kb), it should be possible to achieve reasonable retroviral titres and high level expression of both transgenes. Furthermore, a strategy for conferring resistance to corticosteroids using RNA knockdown of the glutocorticoid receptor NR3C1 is also under investigation in our institute. It may therefore be possible to create a panel of vectors combining resistance to several combinations of immunosuppressive agents, allowing a choice of preferred regimen. Safety concerns regarding a cell product resistant to suppression by several of the main immunosuppressive agents available could be allayed by inclusion of a suicide gene such as iCaspase 9 or HSV-TK.

6.3.2 Lack of immunological niche and requirement for antigen stimulation

Dramatic *in vivo* expansion of *ex vivo* generated EBV-CTLs has been documented following administration post SCT (Rooney*, et al* 1998). One explanation postulated to account for this is the presence of an empty 'immunological niche' as a result of lymphodepletion during pre-transplant conditioning. This lymphodepleted environment persists for many months post stem cell transplant, providing an ideal platform for immunotherapy by eliminating endogenous T cells that compete with the adoptively transferred T cells for cytokines. Lymphodepletion enhances the expansion *in vivo* of adoptively transferred tumour specific T cells in both murine and human studies (Morgan*, et al* 2006, Rapoport*, et al* 2005). In contrast, SOT patients do not undergo conditioning chemotherapy and the level of lymphodepletion achieved by immunosuppression is much less than after SCT. Infused EBV-CTLs must therefore integrate into the established immune environment without an empty niche to fill. It is likely however, that with adequate antigenic stimulation from EBV infected cells, CTLs will proliferate *in vivo* even in the absence of lymphodepletion. Consistent with this, CTLs have proven functional when administered as immunotherapy for neuroblastoma in patients without prior lymphodepletion, therefore it would be expected that EBV-CTLs administered post SOT would also have the capability to expand and persist (Pule*, et al* 2008). In addition, the selective advantage conferred upon CN inhibitor resistant EBV-CTLs in an immunosuppressed environment may

allow preferential expansion and establishment of a long-term EBV-specific compartment.

We have demonstrated that EBV-CTLs remain dependent on repeated antigen stimulation for continued expansion *in vitro*, even with continued supplementation with exogenous IL-2. Critically, no difference was observed in the proliferation of transduced or UT EBV-CTLs in the absence of antigen stimulation, further confirming the safety of CN transduction as increased cellular CN does not provide *de novo* activation signalling. However, these data highlight an important consideration for the efficacy of adoptive immunotherapy with EBV-CTLs, particularly when used as prophylaxis: that the critical driving force for cell expansion both *in vitro* and *in vivo* is presence of specific antigen. Human studies examining transfer of both allodepleted and virus specific T cells have shown that expansion of these T cells occurs after antigen stimulus provided by viral reactivation (Amrolia*, et al* 2006, Cobbold*, et al* 2005) and that without such a stimulus, transferred CTLs do not persist or expand (Leen*, et al* 2006). EBV-CTLs infused to treat established PTLD will receive strong stimulation at the site of tumour and therefore expand in response. This is also likely to be the case when CTLs are given pre-emptively for EBV viraemia. However, in the prophylaxis setting this natural stimulus may be absent. Although B cells expressing EBV proteins are rarely detected in the healthy patient, the natural history of EBV infection involves periodic reactivation of the virus, which may provide occasional antigen specific stimulation *in vivo*. Indeed, this reactivation appears more frequent and prolonged in immunosuppressed patients undergoing SOT. The observed incidence of EBV viraemia in SOT patients depends on the assay used and the patient population studied but appears to be in the range of 40-60% (Benden*, et al* 2005, Lee*, et al* 2005, Martelius*, et al* 2009). Further, it is possible that local, low level, reactivation of EBV in the tonsils and lymph nodes may provide an antigenic stimulus for CTLs even when no viraemia is apparent. Although weekly stimulation is required for cell expansion *in vitro*, it seems that such frequent exposure to antigen is not required *in vivo*, as evidenced by the remarkable long term persistence of EBV-CTLs demonstrated in previous studies (Heslop*, et al* 2009). Gene-marked EBV-CTLs generated using the protocol employed in this study have been detected in peripheral blood of patients many years after infusion, often increasing to in response to a rise in

EBV viral load (Heslop, *et al* 2009, Pule, *et al* 2004). This indicates that memory EBV-CTLs do not require continual antigen exposure to persist *in vivo*, and that they retain the ability to expand in response to the resurgence of antigen. In addition, our phenotyping data reveal a high proportion of central memory cells in transduced EBV-CTL lines, which may enhance the ability of these cell lines to establish a memory population and persist long term *in vivo*.

6.3.2 Culture duration and memory phenotype of CTLs

Many considerations exist concerning the ideal characteristics of *ex vivo* generated T cells for immunotherapy, including the effect of extended culture times and the advantages of administering naïve compared to memory or effector cells. EBV-CTLs administered for the treatment or prevention of PTLD are generally cultured for 4-6 stimulations *ex vivo* to reduce the likelihood of alloreactivity in the CTL line and increase EBV-specificity. However, increasing EBV-specific cytotoxicity by EBV-CTL lines is not correlated with improved *in vivo* function (Haque, *et al* 2007). Indeed a recent study of the efficacy of immunotherapy following *ex vivo* culture suggests that increased stimulation is associated with reduced rather than enhanced functionality, as homing markers drop and cells become further differentiated (Gattinoni, *et al* 2005). Gattinoni *et al* observed reduced efficacy of late effector compared to naïve or early effector cells in a melanoma model, establishing an inverse correlation between *ex vivo* culture and *in vivo* anti-tumour efficacy. The authors suggest that current criteria for selection of T cells for immunotherapy, which currently include the ability to secrete IFN-γ and increased cytotoxicity in response to target cells may not in fact select those cells that are likely to be most efficacious *in vivo*.

Further investigation by Berger *et al* recently reported that antigen-specific effector clones (T_E) derived from central memory (T_{CM}) but not effector memory (T_{EM}) populations *in vitro* are able to revert to T_{CM} *in vivo* following infusion in a macaque CMV model. These cells are then able to persist and are detectable for over 4 months, compared to only days for T_E derived from a T_{EM} population which do not re-express

CD62L, remain committed and undergo apoptosis following infusion (Berger, *et al* 2008a). This indicates that *ex vivo* expansion does not necessarily lead to clonal exhaustion of CTLs when returned to an *in vivo* environment, despite the observed plateau in cell expansion when maintained in culture long term.

Despite these findings, immunotherapy with *ex vivo* expanded EBV-CTLs following an extended period of culture and demonstrating an effector memory phenotype has been very successful for PTLD treatment in the SCT setting. Persistence of such cells has been demonstrated by detection of the neo^R marker gene at up to 85 months post infusion, despite their committed phenotype (Hale, *et al* 2008, Heslop, *et al* 1996). In addition, a recent study of EBV-CTLs transduced with a chimaeric T cell receptor recognising the neuroblastoma epitope GD2 demonstrated that CTLs persist for over 6 weeks in circulation and are capable of expanding in response to stimulation with EBV expressing target cells at 24 weeks. These transduced CTLs uniformly displayed a T_{EM} phenotype at infusion, yet were able to persist *in vivo* following administration. Together, these findings suggest that more committed effector cells are indeed able to persist long term following administration, and that the presence of antigen *in vivo* may overcome the senescence observed both *in vitro* and in some animal models. It should be noted that the study by Gattinoni *et al* utilised a tumour model necessitating active immunisation in addition to T cell transfer, whereas both the animal model utilised by Berger *et al* and the immunotherapy trials in humans were performed in the setting of persistent viral infection which may reflect disparate immunological environments. In conclusion, it is clear that adoptively transferred EBV-CTLs are indeed able to persist long term, mediate anti-viral effects and re-activate in response to antigenic stimulus despite multiple *ex vivo* stimulations with autologous LCL leading to a more committed phenotype.

6.3.3 Immunogenicity of CN mutant transgene

The therapeutic use of transduced cells has been limited in some circumstances by the generation of an immune response to the transgene or vector. This is commonly seen where the transgene is derived from another species. For example seven of 28 patients

treated with herpes simplex virus thymidine kinase (HSV-TK) suicide gene transduced T lymphocytes developed anti-TK CD8+ T cells (Traversari*, et al* 2007, Zaldumbide and Hoeben 2007). Such responses are not restricted to non-human transgenes, indeed rejection of bone marrow grafts as a result of a single amino acid difference in HLA-B44 have been documented (Fleischhauer*, et al* 1990). In addition, alloreactive T cells recognising polymorphic minor histocompatibility antigen mismatches mediate graft versus host disease in HLA-identical sibling SCTs (Goulmy 1996, Goulmy*, et al* 1996). Furthermore, minor histocompatibility antigens may also play a role in rejection after SOT (Dierselhuis and Goulmy 2009). A maximum of three altered amino acids are present in our CN mutants, which minimises the likelihood of such an immune response, however the presence of a novel amino acid sequence may generate neoepitopes and the possibility of an immune response cannot be ruled out. Furthermore, in our approach CN transduced EBV-CTLs will be administered to patients receiving substantial immunosuppressive therapy designed to prevent an allogeneic response, therefore allowing the survival of a foreign organ which is often partially HLA mismatched. With the degree of immunosuppression required to prevent rejection of an allogeneic organ and impairment of the memory EBV immune response, the likelihood of mounting a successful primary immune response towards CN mutants is low.

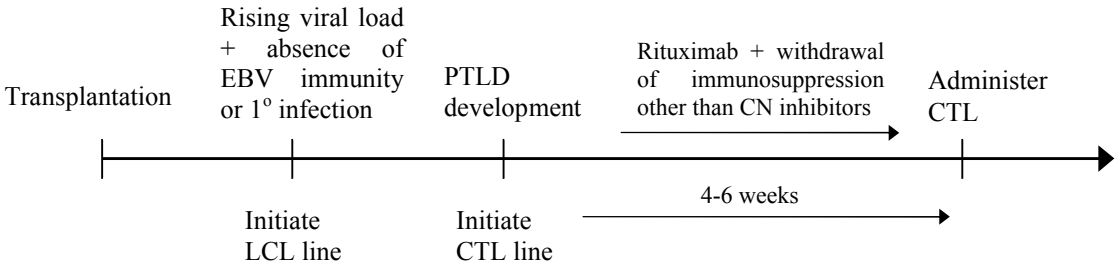
To further investigate the potential immunogenicity of CN mutants, we have used a computer modelling program to predict the likelihood that peptides from the mutant CN containing the altered sequences will be presented by the common HLA alleles HLA-A1, A2, B7 and B8. To this end we have utilised the Bimass calculator (http://www-bimas.cit.nih.gov/molbio/hla_bind/), which predicts the stability of an HLA-peptide complex, and can therefore be used as an indicator of the likelihood that such a complex will be formed. When compared to the stability of the NLV peptide derived from CMV pp65 protein, an immunogenic peptide presented in HLA-A2, most peptides derived from mutant CNs were predicted to have very low stability and therefore are unlikely to be formed. The stability of NLV in HLA-A*0201 is 160 units, whereas most CN peptides containing mutant sequences had a stability of under 25U. However, one peptide derived from CNa12 beginning at amino acid 346 with the sequence FMDVFEWSA contained both amino acid changes introduced into CNa12

and is predicted to have a binding efficiency of 188U. Although this indicates that the HLA-A*0201: FMDVFEWSA complex will be relatively stable, this stability is in fact much reduced when compared to the same peptide from the wtCNa sequence, FMDVFTWSL, which has a predicted stability of 809U. In addition, three CN peptides from CNa12 not containing any mutations have higher predicted stabilities than FMDVFEWSA of between 1055U and 201U. Therefore, when considered in the context of the other peptides available for presentation it is likely that CNa12 FMDVFEWSA will not be the predominant peptide. However, it is possible that peptides derived from CN mutants can indeed be bound and presented by the common HLA alleles; therefore the possibility of triggering an immune response cannot be ruled out even in the presence of immunosuppression. In subsequent clinical studies it will be important to assess the *in vivo* survival of CN mutant transduced CTLs and to examine if any cellular response against the transgene occurs.

6.3.4 Practical considerations

Finally, a major obstacle to the more widespread use of adoptive immunotherapy in many settings is the considerable time, expertise and specialist facilities required for generation of the cell products. This is especially the case where autologous cells are generated on a patient-by-patient basis. Therefore, the appropriate clinical setting to gain maximal benefit from adoptive immunotherapy must be carefully chosen. Initially, focussing this approach on those patients likely to gain maximum benefit is necessary, and targeting of very high risk groups is a rational approach. Infusion of autologous CN inhibitor resistant EBV specific CTLs may provide effective prophylaxis for PTLD, for example in patients undergoing paediatric small bowel transplantation, where the risk of PTLD may be as high as 20% (Thompson 1999). In lower risk patient groups where prophylaxis is more difficult to justify, CTLs could be used as an adjunctive therapy for established PTLD. As illustrated below, we propose a possible strategy whereby EBV viral load is closely monitored, with initiation of an LCL line when rising loads are detected (Savoldo*, et al* 2006). Depending on resources, this could either be done for all patients with rising viral loads, or restricted to those at highest risk, for example those experiencing primary EBV infection or

where no EBV-specific immunity is detected. Upon development of PTLD, generation of an EBV-CTL line begins concurrent with withdrawal of non-CN inhibitor immunosuppression and administration of Rituximab to control disease. After generation and testing, EBV-CTLs are administered to maintain remission induced by first line therapy, or treat remaining disease. This approach will allow targeted production of CTL lines for those patients most likely to develop PTLD, in addition to circumventing the high risk of partial response and relapse associated with Rituximab monotherapy (Choquet*, et al* 2006, Choquet*, et al* 2007, Messahel*, et al* 2006).



Alternatively, CN inhibitor resistant EBV-CTLs could represent a therapeutic approach for patients relapsing following first line Rituximab therapy as these patients have a poor prognosis. Two studies examining the efficacy of Rituximab therapy for PTLD have demonstrated that various parameters including abnormal lactate dehydrogenase levels, multivisceral disease, CNS involvement and late onset of lymphoma predispose to a poor response to Rituximab, therefore early initiation of a CTL line might be appropriate in such patients (Benkerrou*, et al* 1998, Choquet*, et al* 2006).

Targeted generation of EBV-CTL lines for patients identified as likely to develop PTLD would be the ideal approach to immunotherapy for this disease. However, the prediction of individual patients at highest risk for PTLD development has been problematic following SOT. The most accurate prediction methods to date utilise measures of both EBV-specific immunity and EBV viral load, including the I_{PTLD} index developed by Smets *et al* (Smets*, et al* 2002) and the protocol developed by

Meij *et al* utilising EBV-tetramers to measure EBV immunity (Meij, *et al* 2003). These methods are not yet in clinical use and present some practical difficulties such as the generation of an LCL line for the I_{PTLD}. Advances in the measurement and interpretation of EBV loads possibly in combination with alternative measures of general, rather than EBV-specific immunity may allow more accurate identification of the highest risk patients in future (Baldanti, *et al* 2000, Lee, *et al* 2006a, Sebelin-Wulf, *et al* 2007). This will allow focussing of resources on those patients most likely to require cellular therapy, both increasing cost-effectiveness and allowing early initiation of cell lines to reduce the delay between diagnosis and treatment.

6.4 Further work and scale-up studies

6.4.1 Further in vitro investigations

Additional investigations to further examine the effect of transduction with CN mutants would strengthen this study. Assessment of Vβ gene usage by spectratyping before and after transduction would allow determination of whether expression of CN mutants skews the T cell repertoire (Coito, *et al* 2004, Ferrand, *et al* 2000, Recchia, *et al* 2006). Assessment following several weeks culture in CN inhibitors would determine whether this selective pressure results in the preferential outgrowth of a particular clone.

Examination of phenotypic markers suggest that this is unlikely to be the case (chapter 4, figure 43), nevertheless demonstration of balanced Vβ usage after transduction and culture in CN inhibitors would be of benefit in the progress towards scale-up work and subsequent clinical trial. In addition, although transduction with RV does not lead to inappropriate proliferation of mature T cells (Recchia, *et al* 2006), identification of integration copy number and sites within transduced EBV-CTL lines would provide useful safety data prior to proceeding to clinical studies.

6.4.2 Scale-up studies

EBV-CTLs have been generated on a large scale for use in many clinical trials as discussed previously. However, scale-up studies are necessary for generation of large numbers of CN transduced EBV-CTLs under conditions of Good Manufacturing Practice (GMP). GMP production of LCL lines presents practical difficulties as it requires the use of EBV virus produced from the B95-8 cell line. As EBV from B95-8 supernatant is a replication competent virus, it must be handled in a separate GMP facility to that utilised for EBV-CTL production. Therefore, LCL lines would need to be generated in one facility, irradiated and transferred to another GMP laboratory used for EBV-CTL generation. In addition, larger numbers of EBV-CTLs would need to be generated to provide sufficient cells for administration to patients compared to that needed for these *in vitro* experiments: In prior clinical studies with EBV-CTLs, cell doses of between $2x10^7$ and $5x10^8$ cells/m^2 have been administered. This may be facilitated by use of the Wilson Wolf GP500 bioreactor for large scale production of CTLs, which has been shown to improve the growth profile of EBV-CTL lines *in vitro* without any apparent detrimental effect on CTL function (Vera*, et al* 2008). The production of a larger number of cells in a shorter space of time may also partly address the considerable lag time between initiation of cell lines and the availability of the therapeutic product. However, shortening the process of CTL generation will have limited impact on the overall time required to generate the cell product because of the need for first establishing a patient-specific LCL stimulator line. Improved EBV-CTL expansion in bioreactors is likely to reduce the time from LCL initiation to availability of EBV-CTLs from approximately 8-10 to 6-8 weeks. Until the production of LCLs can be achieved in a shorter space of time, or effective alternatives such as artificial antigen presenting cells are developed, the generation of EBV-CTLs will be a lengthy process.

One alternative to LCLs is the use of peptide pools which can be loaded onto APCs such as dendritic cells, and used to stimulate effector cells; however this would necessitate the use of much larger volumes of blood than for LCL generation. Such peptide pools have been produced for various EBV proteins, including the Peptivator mixes (Miltenyi) which cover many epitopes derived from the lytic BZLF-1 or latent

EBNA-1 proteins. Unfortunately, as discussed in section 1.1.4, CD8+ EBV-latency specific effector cells recognise epitopes mainly derived from EBNA-3 proteins, therefore these peptide mixes are not yet optimal for use generating the predominantly CD8 EBV-CTL lines necessary for PTLD treatment.

6.4.3 In vivo studies

Following successful demonstration of CN inhibitor resistance *in vitro*, examination of the safety and efficacy of this approach in an *in vivo* model is necessary as a prelude to clinical studies. In this study, the establishment of a xenogenic γ-chain$^{-/-}$/RAG2$^{-/-}$/C5$^{-/-}$ mouse model was undertaken. Successful engraftment and growth of subcutaneous LCL tumours was demonstrated, along with bioluminescent imaging for accurate monitoring of tumour growth. However, although reduction of LCL tumours following administration of EBV-CTLs was achieved in one set of experiments, this was not consistently seen and further optimisation of this system is required in order to obtain robust data. Potential approaches to this have been discussed in chapter 5. Briefly, we plan to optimise the procedure to reduce unnecessary delays, examine the effect of altering the cytokines added both to CTLs prior to and following infusion, as well as studying the distribution of EBV-CTLs post infusion to establish that appropriate homing is occurring *in vivo*.

Examination of the effect of transduced EBV-CTLs *in vivo* in this animal model will enable assessment of both the safety and efficacy of our approach. Following CTL infusion, several safety aspects will be monitored including toxicity to non-haematopoietic tissues, reactions at the tumour site and any other adverse effects on the animal. The tumour site will be closely monitored for signs of inflammation as a result of cell expansion and, if present, the degree to which this is observed will be compared between mice receiving transduced and non-transduced CTLs in both the presence and absence of CN inhibitors. In addition, mice will be carefully monitored for signs of CTL proliferation at alternative sites, which may indicate autonomous proliferation of the infused cells. At termination of the experiment, multiple tissues will be harvested and examined histologically for signs of CTL persistence, toxicity or

inflammation. In addition to this rigorous safety data, biological efficacy will also be examined in the setting of both prophylaxis, with infusion of EBV-CTLs concurrent with LCL injection, and treatment, with administration of EBV-CTLs following establishment of the LCL tumour. The progress of tumour growth will be carefully monitored and compared to a non-CTL treated cohort to determine if administration of transduced EBV-CTLs results in tumour regression in the presence of CN inhibitors.

However, the use of a murine model to examine the behaviour of human CTLs and LCL has limitations that must be considered when interpreting the obtained data. For both safety and efficacy data, it must be remembered that although APCs are present in the form of a subcutaneous LCL tumour, this model utilises human cells in a murine environment. Despite the provision of recombinant human IL-2, the full cytokine milieu present in human subjects is not recreated in this animal model. In addition, homing molecules and co-stimulatory molecules are of murine origin and may not provide the optimal signals for human CTLs. Therefore this model represents a considerable simplification of the human *in vivo* environment. Despite these limitations, this model has been successfully utilised for the study of PTLD immunotherapy and we believe it to be the most appropriate model available. The examination of both transduced and untransduced EBV-CTLs in the presence and absence of CN inhibitors in this mouse model, combined with long-term follow up of the animals is expected to provide essential data regarding both efficacy and safety of these CTLs *in vivo*. These data will form the basis of the Investigational Medicinal Product (IMP) dossier for application to both the regulatory (MHRA) and ethical (GTAC) bodies prior to commencement of a clinical trial.

6.6 Clinical applications

6.6.1 Clinical trial in paediatric solid organ transplantation

In conjunction with the paediatric transplant teams at Great Ormond Street Hospital (GOSH), our laboratory is working towards the initiation of a clinical trial of CN inhibitor resistant autologous EBV-CTLs in these patients. Paediatric transplant

recipients experience a higher rate of PTLD development than adult patients as a result of higher proportions of EBV naïve patients pre-transplant. Therefore this patient group represent an ideal population in which to study the treatment of PTLD with immunotherapy. We aim to establish a phase I/II trial to examine safety and biological efficacy of transduced EBV-CTL lines for the treatment of PTLD despite ongoing immunosuppression. This clinical trial will include GOSH patients undergoing paediatric small bowel transplantation at King's Hospital, London, as well as those undergoing kidney, heart and heart and lung transplantation at GOSH, with a predicted 60 patients to be transplanted per year, and an average of five patients per year developing PTLD.

We envisage that this trial will examine the efficacy of CNb30 transduced EBV-CTL as adjunctive therapy to reduction in immunosuppression and treatment with Rituximab, as described previously. An LCL line will be initiated upon diagnosis of PTLD, followed by a CTL line as soon as the LCL line is established. Patients will be treated with Rituximab and reduced immunosuppression during this period. CTL lines will be transduced with retrovirus carrying CNb30 to confer resistance to FK506 or CsA, and cryopreserved. It is anticipated that a single dose of $2x10^8$ transduced EBV-CTLs per m^2 will be infused. Patients will be monitored closely to ensure that cell administration is well tolerated and followed up with attention to several end points. The primary end points will include toxicity to the graft and other recipient tissues and assessment of adverse reactions to cell administration. In addition, the circulating frequency of EBV-specific cells will be monitored before and after infusion to determine if an increase in CTL precursor frequency is observed as a result of this treatment. Secondary end points for this study will include examination of the long term persistence of EBV-CTLs *in vivo* as well as the incidence of relapse observed in this treatment group compared to the previously observed 57% relapse following Rituximab monotherapy (Choquet*, et al* 2007).

It is anticipated that infusion of EBV-CTLs will be well tolerated as such cells have previously been administered to over 55 patients following SOT (Comoli*, et al* 2002, Comoli*, et al* 2005, Haque*, et al* 2007, Khanna*, et al* 1999, Savoldo*, et al* 2006). As indicated by the data obtained in this study, transduction of EBV-CTL lines should

allow efficient cell function despite the continuation of ongoing immunosuppression with CN inhibitors, therefore clearing remaining disease as well as contributing to the long term control of PTLD and reducing the relapse rates associated with Rituximab monotherapy.

6.6.2 Alternative applications

Generation of cells that are resistant to CN inhibitors is not limited to use in autologous EBV-CTL lines post SOT. As discussed in section 1.4.2.3, in response to the practical problems of producing autologous EBV-CTLs, an allogeneic EBV-CTL bank has been established, the use of cells from which has induced clinical responses in over 50% of patients failing conventional treatment. The establishment of this CTL bank enables rapid access to immunotherapy for treatment of patients developing PTLD (Haque*, et al* 2007). Despite these promising results, 48% of patients do not maintain responses at six months post infusion and this may be due to either the continuation of immunosuppressive therapy or the generation of an immune response by the recipient against the inevitable mismatches. Transduction of third party EBV-CTLs from this cell bank with CN mutants conferring CN inhibitor resistance would circumvent the former problem and may allow longer term persistence. In addition, our approach would allow the continuation of immunosuppressive therapy with CN inhibitors at therapeutic levels, potentially reducing the likelihood of an allo-response against the infused cells.

In the stem cell transplant setting, the application of immunotherapy has widened in recent years to include the treatment of an increasing number of viruses. Immunotherapy with CMV-specific CTLs generated by repetitive stimulation has been used to treat CMV viraemia post SCT. This approach has been shown to augment circulating CMV-specific responses and shows some efficacy for treating CMV reactivation in patients undergoing SCT from matched sibling donors. In the majority of cases, immunosuppression with CN inhibitors was not withdrawn in these studies. It is possible that CMV-specific CTLs are less susceptible to the effects of CN inhibitors than EBV-specific CTLs, but it is difficult to assess the true efficacy of adoptively

transferred CTLs in the matched sibling donor setting where recovery of CMV-specific T cells infused with the stem cell graft occurs early. Similarly, using LCL transduced with an adenoviral vector carrying the pp65 transgene as antigen presenting cells, we and others have generated CTLs directed against EBV, CMV and adenovirus specific T cells in a single culture for protection from the three main viral threats post SCT (Karlsson*, et al* 2007, Leen*, et al* 2006). As in the SOT setting, transduction of virus-specific CTLs with our CN mutants may enable improved virus-specific immunity without the need for early withdrawal of CN inhibitors and the consequent risk of GVHD. Furthermore, viral reactivations are frequently associated with immunosuppressive therapy for GVHD, and the use of CN inhibitor resistant CTLs in this setting is an attractive option. In this regard, it would be even more useful to combine resistance to CN inhibitors and steroids.

Recently developed techniques such as the Miltenyi γ-capture system enable stimulation with viral-antigen presenting APCs and subsequent selection of responders to enrich for virus specific cells. Similarly, virus-specific CD8+ T cells can be isolated from seropositive donors using HLA-peptide multimers (Cobbold*, et al* 2005). These approaches are being investigated for the pre-emptive treatment of both adenovirus and CMV infections post SCT, and could be combined with CNb30 transduction to allow effective function despite ongoing GVHD prophylaxis (Cobbold*, et al* 2005, Feuchtinger*, et al* 2008, Peggs*, et al* 2003).

Another approach to provide protection from multiple pathogens is selective depletion of alloreactive donor lymphocytes and infusion of the remaining non-alloreactive cells to reconstitute T cell immunity. In this approach, donor T cells are cultured with recipient APCs to activate allo-reactive T cells which can then be selectively depleted using antibodies that recognise activation markers such as CD25, or photodynamic purging. This ensures that allo-reactive cells most likely to cause GVHD are removed and the remaining allo-depleted population of donor cells can be safely infused with a low incidence of GVHD despite increased cell doses. Allodepleted T cells have been shown to safely augment immune reconstitution after both matched sibling and haploidentical SCT (Amrolia*, et al* 2006, Mielke*, et al* 2008). These approaches could be combined with CN inhibitor resistance as an additional safety measure to allow

efficient function of infused cells following administration into a patient receiving immunosuppressive therapy for GVHD prophylaxis.

Finally, the use of anti-leukaemic T cells following SCT to remove residual malignant cells and ensure long term remission has been explored in recent years. Indeed, particularly in myeloid malignancies, the graft-versus leukaemia (GVL) activity of T cells infused with the stem cell graft may play a significant role in curing haematopoietic malignancies, rather than myeloablation and subsequent replacement of the haematopoietic system itself (Feng*, et al* 2008). It is clear that for some malignancies, immunotherapy with donor lymphocyte infusions to augment anti-leukaemic activity post SCT can be effective in preventing and treating relapse after SCT (Bader*, et al* 2004, van Rhee and Kolb 1995). There is strong evidence to suggest that minor histocompatibility antigen (mHAg) mismatches between the stem cell donor and the recipient can be the target of either GVHD or GVL responses post SCT (Feng*, et al* 2008). Certain mHAgs such as HA-1 and HA-2 are expressed predominantly on haematopoietic tissues and as such provide an ideal target for enhancement of the GVL effect without the concurrent risk of increasing GVHD. Anti-mHAg CTL clones have been identified in the circulation of patients receiving donor lymphocyte infusions and correlated with the occurrence of remission. Additionally, such cells have been demonstrated to exhibit anti-leukaemic activity in *in vitro* assays (Marijt*, et al* 2003). Therefore immunotherapy with *ex vivo* expanded mHAg-specific T cells holds considerable promise for the treatment of haematological malignancies in the HLA-matched, mHAg mismatched SCT setting. Using retroviral transfer of cDNAs encoding the TCR α and β genes, it is possible to re-direct T cells to a desired specificity and the generation of anti-HA-2 specific T cells for clinical use via this method is now possible. Although the applicability of this approach may be limited by the need for the appropriate HLA-restriction and mismatched mHAg expression by the host, potentially this is an attractive option for this subset of patients.

The ability to confer specificity towards particular epitopes on T cells by TCR transfer has allowed the generation of cells directed towards other leukaemia antigens for immunotherapy. This approach has already been applied to non-haematological malignant disorders including the first clinical trial by the Rosenberg group utilising

MART-1 TCR transgenic CD8+ T cells, which successfully induced tumour regression in two of fifteen patients with refractory metastatic melanoma (Morgan*, et al* 2006). Another candidate for immunotherapy with TCR transduced T cells is the WT-1 antigen, which is overexpressed in both acute and chronic myeloid leukaemias, myelodysplastic syndrome and some solid tumours but displays low physiological expression in a limited range of tissues. Anti-WT-1 T cells have been generated using lentiviral vectors (Stauss*, et al* 2008) and shown to eliminate human leukaemia cells in an *in vivo* NOD/SCID mouse model (Xue*, et al* 2005, Xue*, et al* 2009).

Further developments in the transfer of TCRs to generate T cells with a particular specificity have resulted in the generation of chimaeric antigen receptors (CAR), which combine the specificity of an antibody binding domain with signalling regions from the TCR, thus removing the need for HLA-matching of the introduced TCR and the patient. This approach has proved successful when applied to the treatment of neuroblastoma, with half of subjects demonstrating tumour regression or necrosis following infusion of EBV-CTLs transduced with an anti-diasialoganglioside GD2 CAR (Pule*, et al* 2008).

Many of the approaches described above may have greater efficacy when applied early following transplantation in the setting of minimal residual disease. However patients are often treated prophylactically with immunosuppression in the immediate post-transplant period to prevent the development of graft-versus-host disease (GVHD). Particularly for anti-tumour therapies, this early window would be ideal for the application of immunotherapy as a result of the low tumour burden and empty lymphoid compartment at this time allowing cell expansion. The use of CN inhibitor resistant CTLs would allow continued protection from GVHD with immunosuppression, concurrent with administration of an effective cell product.

6.7 Conclusions

We report the generation of CN mutants capable of conferring resistance to CN inhibitors in cell lines and primary EBV-CTLs. The development of CN inhibitor resistant EBV-CTLs may represent a considerable advance in the management of PTLD, particularly for high risk patients. Reconstitution of effective EBV-specific immunity without the requirement for a reduction in immunosuppressive therapy should allow the prevention or treatment of EBV-driven B cell proliferation, in conjunction with continued protection of the grafted organ. Establishment of a memory population of CN inhibitor resistant EBV-specific cells may result in effective long-term surveillance and protection from relapse along with unimpaired graft function. In addition, the wider application of this approach may allow more effective T cell immunotherapy for viral infections and malignancy post stem cell transplantation.

References

Abu-Elmagd, K., Reyes, J., Todo, S., Rao, A., Lee, R., Irish, W., Furukawa, H., Bueno, J., McMichael, J., Fawzy, A.T., Murase, N., Demetris, J., Rakela, J., Fung, J.J. & Starzl, T.E. (1998) Clinical intestinal transplantation: new perspectives and immunologic considerations. *J Am Coll Surg,* **186,** 512-525; discussion 525-517.

Abu-Elmagd, K.M., Mazariegos, G., Costa, G., Soltys, K., Bond, G., Sindhi, R., Green, M., Jaffe, R., Wu, T., Koritsky, D., Matarese, L., Schuster, B., Martin, L., Dvorchik, I. & Nalesnik, M.A. (2009) Lymphoproliferative disorders and de novo malignancies in intestinal and multivisceral recipients: improved outcomes with new outlooks. *Transplantation,* **88,** 926-934.

Ahmad, I., Cau, N.V., Kwan, J., Maaroufi, Y., Meuleman, N., Aoun, M., Lewalle, P., Martiat, P., Crokaert, F. & Bron, D. (2009) Preemptive management of Epstein-Barr virus reactivation after hematopoietic stem-cell transplantation. *Transplantation,* **87,** 1240-1245.

Alak, A.M. & Lizak, P. (1996) Stability of FK506 in blood samples. *Ther Drug Monit,* **18,** 209-211.

Amrolia, P.J., Muccioli-Casadei, G., Huls, H., Adams, S., Durett, A., Gee, A., Yvon, E., Weiss, H., Cobbold, M., Gaspar, H.B., Rooney, C., Kuehnle, I., Ghetie, V., Schindler, J., Krance, R., Heslop, H.E., Veys, P., Vitetta, E. & Brenner, M.K. (2006) Adoptive immunotherapy with allodepleted donor T-cells improves immune reconstitution after haploidentical stem cell transplantation. *Blood,* **108,** 1797-1808.

Aringer, M. (2002) T lymphocyte activation--an inside overview. *Acta Med Austriaca,* **29,** 7-13.

Bader, P., Kreyenberg, H., Hoelle, W., Dueckers, G., Handgretinger, R., Lang, P., Kremens, B., Dilloo, D., Sykora, K.W., Schrappe, M., Niemeyer, C., Von Stackelberg, A., Gruhn, B., Henze, G., Greil, J., Niethammer, D., Dietz, K., Beck, J.F. & Klingebiel, T. (2004) Increasing mixed chimerism is an important prognostic factor for unfavorable outcome in children with acute lymphoblastic leukemia after allogeneic stem-cell transplantation: possible role for pre-emptive immunotherapy? *J Clin Oncol,* **22,** 1696-1705.

Baldanti, F., Grossi, P., Furione, M., Simoncini, L., Sarasini, A., Comoli, P., Maccario, R., Fiocchi, R. & Gerna, G. (2000) High levels of Epstein-Barr virus DNA in blood of solid-organ transplant recipients and their value in predicting posttransplant lymphoproliferative disorders. *J Clin Microbiol,* **38,** 613-619.

Benden, C., Aurora, P., Burch, M., Cubitt, D., Lloyd, C., Whitmore, P., Neligan, S.L. & Elliott, M.J. (2005) Monitoring of Epstein-Barr viral load in pediatric heart and lung transplant recipients by real-time polymerase chain reaction. *J Heart Lung Transplant,* **24,** 2103-2108.

Benkerrou, M., Jais, J.-P., Leblond, V., Durandy, A., Sutton, L., Bordigoni, P., Garnier, J.L., Le Bidois, J., Le Deist, F., Blanche, S. & Fischer, A. (1998) Anti-B-Cell Monoclonal Antibody Treatment of Severe Posttransplant B-Lymphoproliferative Disorder: Prognostic Factors and Long-Term Outcome. *Blood,* **92,** 3137-3147.

Berger, C., Jensen, M.C., Lansdorp, P.M., Gough, M., Elliott, C. & Riddell, S.R. (2008a) Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest,* **118,** 294-305.

Berger, F., Paulmurugan, R., Bhaumik, S. & Gambhir, S.S. (2008b) Uptake kinetics and biodistribution of 14C-D-luciferin--a radiolabeled substrate for the firefly luciferase catalyzed bioluminescence reaction: impact on bioluminescence based reporter gene imaging. *Eur J Nucl Med Mol Imaging,* **35,** 2275-2285.

Birkeland, S.A. (1983) Chronic antigenic stimulation from the graft as a possible oncogenic factor after renal transplant. *Scand J Urol Nephrol,* **17,** 355-359.

Birkeland, S.A. & Hamilton-Dutoit, S. (2003) Is posttransplant lymphoproliferative disorder (PTLD) caused by any specific immunosuppressive drug or by the transplantation per se? *Transplantation,* **76,** 984-988.

Blaese, R.M., Culver, K.W., Miller, A.D., Carter, C.S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., Greenblatt, J.J., Rosenberg, S.A., Klein, H., Berger, M., Mullen, C.A., Ramsey, W.J., Muul, L., Morgan, R.A. & Anderson, W.F. (1995) T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. *Science,* **270,** 475-480.

Bosma, G.C., Custer, R.P. & Bosma, M.J. (1983) A severe combined immunodeficiency mutation in the mouse. *Nature,* **301,** 527-530.

Branchini, B.R., Ablamsky, D.M., Murtiashaw, M.H., Uzasci, L., Fraga, H. & Southworth, T.L. (2007) Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications. *Anal Biochem,* **361,** 253-262.

Brumbaugh, J., Baldwin, J.C., Stinson, E.B., Oyer, P.E., Jamieson, S.W., Bieber, C.P., Henle, W. & Shumway, N.E. (1985) Quantitative analysis of immunosuppression in cyclosporine-treated heart transplant patients with lymphoma. *J Heart Transplant,* **4,** 307-311.

Buell, J.F., Gross, T.G. & Woodle, E.S. (2005) Malignancy after transplantation. *Transplantation,* **80,** S254-264.

Caillard, S., Dharnidharka, V., Agodoa, L., Bohen, E. & Abbott, K. (2005) Posttransplant Lymphoproliferative Disorders after Renal Transplantation in the United States in Era of Modern Immunosuppression. *Transplantation,* **80,** 1233-1243.

Callan, M.F.C., Tan, L., Annels, N., Ogg, G.S., Wilson, J.D.K., O'Callaghan, C.A., Steven, N., McMichael, A.J. & Rickinson, A.B. (1998) Direct Visualization of Antigen-specific CD8+ T Cells during the Primary Immune Response to Epstein-Barr Virus In Vivo. *J. Exp. Med.,* **187,** 1395-1402.

Cardenas, M.E., Muir, R.S., Breuder, T. & Heitman, J. (1995) Targets of immunophilin-immunosuppressant complexes are distinct highly conserved regions of calcineurin A. *Embo J,* **14,** 2772-2783.

Chandran, S., Williams, S. & Denmeade, S. (2009) Extended-release PEG-luciferin allows for long-term imaging of firefly luciferase activity <I>in vivo</I>. *Luminescence,* **24,** 35-38.

Chatila, T., Ho, N., Liu, P., Liu, S., Mosialos, G., Kieff, E. & Speck, S.H. (1997) The Epstein-Barr virus-induced Ca2+/calmodulin-dependent kinase type IV/Gr promotes a Ca(2+)-dependent switch from latency to viral replication. *J Virol,* **71,** 6560-6567.

Choquet, S., Leblond, V., Herbrecht, R., Socie, G., Stoppa, A.M., Vandenberghe, P., Fischer, A., Morschhauser, F., Salles, G., Feremans, W., Vilmer, E., Peraldi, M.N., Lang, P., Lebranchu, Y., Oksenhendler, E., Garnier, J.L., Lamy, T., Jaccard, A., Ferrant, A., Offner, F., Hermine, O., Moreau, A., Fafi-Kremer, S., Morand, P., Chatenoud, L., Berriot-Varoqueaux, N., Bergougnoux, L. & Milpied, N. (2006) Efficacy and safety of rituximab in B-cell post-transplantation lymphoproliferative disorders: results of a prospective multicenter phase 2 study. *Blood,* **107,** 3053-3057.

Choquet, S., Oertel, S., LeBlond, V., Riess, H., Varoqueaux, N., Dorken, B. & Trappe, R. (2007) Rituximab in the management of post-transplantation lymphoproliferative disorder after solid organ transplantation: proceed with caution. *Ann Hematol,* **86,** 599-607.

Ciceri, F., Bonini, C., Marktel, S., Zappone, E., Servida, P., Bernardi, M., Pescarollo, A., Bondanza, A., Peccatori, J., Rossini, S., Magnani, Z., Salomoni, M., Benati, C., Ponzoni, M., Callegaro, L., Corradini, P., Bregni, M., Traversari, C. & Bordignon, C. (2007) Antitumor effects of HSV-TK engineered donor lymphocytes after allogeneic stem-cell transplantation. *Blood,* **109,** 4698-4707.

Ciceri, F., Bonini, C., Stanghellini, M.T.L., Bondanza, A., Traversari, C., Salomoni, M., Turchetto, L., Colombi, S., Bernardi, M., Peccatori, J., Pescarollo, A., Servida, P., Magnani, Z., Perna, S.K., Valtolina, V., Crippa, F., Callegaro, L., Spoldi, E., Crocchiolo, R., Fleischhauer, K., Ponzoni, M., Vago, L., Rossini, S., Santoro, A., Todisco, E., Apperley, J., Olavarria, E., Slavin, S., Weissinger, E.M., Ganser, A., Stadler, M., Yannaki, E., Fassas, A., Anagnostopoulos, A.,

Bregni, M., Stampino, C.G., Bruzzi, P. & Bordignon, C. (2009) Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *The Lancet Oncology,* **10,** 489-500.

Cobbold, M., Khan, N., Pourgheysari, B., Tauro, S., McDonald, D., Osman, H., Assenmacher, M., Billingham, L., Steward, C., Crawley, C., Olavarria, E., Goldman, J., Chakraverty, R., Mahendra, P., Craddock, C. & Moss, P.A. (2005) Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *J Exp Med,* **202,** 379-386.

Cockfield, S.M. (2001) Identifying the patient at risk for post-transplant lymphoproliferative disorder. *Transpl Infect Dis,* **3,** 70-78.

Cohen, J.I. (2000) Epstein-Barr virus infection. *N Engl J Med,* **343,** 481-492.

Coito, S., Sauce, D., Duperrier, A., Certoux, J.M., Bonyhadi, M., Collette, A., Kuehlcke, K., Herve, P., Tiberghien, P., Robinet, E. & Ferrand, C. (2004) Retrovirus-mediated gene transfer in human primary T lymphocytes induces an activation- and transduction/selection-dependent TCR-B variable chain repertoire skewing of gene-modified cells. *Stem Cells Dev,* **13,** 71-81.

Comoli, P., Ginevri, F., Maccario, R., Frasson, C., Valente, U., Basso, S., Labirio, M., Huang, G.C., Verrina, E., Baldanti, F., Perfumo, F. & Locatelli, F. (2006) Successful In Vitro Priming of EBV-Specific CD8+ T Cells Endowed with Strong Cytotoxic Function from T Cells of EBV-Seronegative Children. *American Journal of Transplantation,* **6,** 2169-2176.

Comoli, P., Labirio, M., Basso, S., Baldanti, F., Grossi, P., Furione, M., Vigano, M., Fiocchi, R., Rossi, G., Ginevri, F., Gridelli, B., Moretta, A., Montagna, D., Locatelli, F., Gerna, G. & Maccario, R. (2002) Infusion of autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication. *Blood,* **99,** 2592-2598.

Comoli, P., Maccario, R., Locatelli, F., Valente, U., Basso, S., Garaventa, A., Tomà, P., Botti, G., Melioli, G., Baldanti, F., Nocera, A., Perfumo, F. & Ginevri, F. (2005) Treatment of EBV-Related Post-Renal Transplant Lymphoproliferative Disease with a Tailored Regimen Including EBV-Specific T Cells. *American Journal of Transplantation,* **5,** 1415-1422.

Contag, C.H., Spilman, S.D., Contag, P.R., Oshiro, M., Eames, B., Dennery, P., Stevenson, D.K. & Benaron, D.A. (1997) Visualizing gene expression in living mammals using a bioluminescent reporter. *Photochem Photobiol,* **66,** 523-531.

Contag, P.R., Olomu, I.N., Stevenson, D.K. & Contag, C.H. (1998) Bioluminescent indicators in living mammals. *Nat Med,* **4,** 245-247.

Contassot, E., Robinet, E., Angonin, R., Laithier, V., Bittencourt, M., Pavy, J.J., Cahn, J.Y., Herve, P. & Tiberghien, P. (1998) Differential effects of cyclosporin A on the alloreactivity of fresh and ex vivo-expanded T lymphocytes. *Bone Marrow Transplant,* **22,** 1097-1102.

Craig, F.E., Johnson, L.R., Harvey, S.A., Nalesnik, M.A., Luo, J.H., Bhattacharya, S.D. & Swerdlow, S.H. (2007) Gene expression profiling of Epstein-Barr virus-positive and -negative monomorphic B-cell posttransplant lymphoproliferative disorders. *Diagn Mol Pathol,* **16,** 158-168.

Crumpacker, C.S. (1996) Ganciclovir. *N Engl J Med,* **335,** 721-729.

Darenkov, I.A., Marcarelli, M.A., Basadonna, G.P., Friedman, A.L., Lorber, K.M., Howe, J.G., Crouch, J., Bia, M.J., Kliger, A.S. & Lorber, M.I. (1997) Reduced incidence of Epstein-Barr virus-associated posttransplant lymphoproliferative disorder using preemptive antiviral therapy. *Transplantation,* **64,** 848-852.

Davis, C.L. (1998) The antiviral prophylaxis of post-transplant lymphoproliferative disorder. *Springer Semin Immunopathol,* **20,** 437-453.

Davis, T.A., White, C.A., Grillo-Lopez, A.J., Velasquez, W.S., Link, B., Maloney, D.G., Dillman, R.O., Williams, M.E., Mohrbacher, A., Weaver, R., Dowden, S. & Levy, R. (1999) Single-agent monoclonal antibody efficacy in bulky non-Hodgkin's lymphoma: results of a phase II trial of rituximab. *J Clin Oncol,* **17,** 1851-1857.

Dierselhuis, M. & Goulmy, E. (2009) The relevance of minor histocompatibility antigens in solid organ transplantation. *Curr Opin Organ Transplant,* **14,** 419-425.

Dilloo, D., Bacon, K., Holden, W., Zhong, W., Burdach, S., Zlotnik, A. & Brenner, M. (1996) Combined chemokine and cytokine gene transfer enhances antitumor immunity. *Nat Med,* **2,** 1090-1095.

Doesch, A.O., Konstandin, M., Celik, S., Kristen, A., Frankenstein, L., Sack, F.U., Schnabel, P., Schnitzler, P., Katus, H.A. & Dengler, T.J. (2008) Epstein-Barr virus load in whole blood is associated with immunosuppression, but not with post-transplant lymphoproliferative disease in stable adult heart transplant patients. *Transpl Int,* **21,** 963-971.

Einsele, H., Roosnek, E., Rufer, N., Sinzger, C., Riegler, S., Loffler, J., Grigoleit, U., Moris, A., Rammensee, H.-G., Kanz, L., Kleihauer, A., Frank, F., Jahn, G. & Hebart, H. (2002) Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood,* **99,** 3916-3922.

Elstrom, R.L., Andreadis, C., Aqui, N. & Tsai, D.E. (2005) Treatment of Post-Transplant Lymphoproliferative Disorder (PTLD) with Rituximab or

Chemotherapy: The University of Pennsylvania Experience. *ASH Annual Meeting Abstracts,* **106,** 936-.

Engstrand, M., Lidehall, A.K., Totterman, T.H., Herrman, B., Eriksson, B.M. & Korsgren, O. (2003) Cellular responses to cytomegalovirus in immunosuppressed patients: circulating CD8+ T cells recognizing CMVpp65 are present but display functional impairment. *Clin Exp Immunol,* **132,** 96-104.

Epstein, M.A., Achong, B.G. & Barr, Y.M. (1964) Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. *Lancet,* **1,** 702-703.

Everly, M.J., Bloom, R.D., Tsai, D.E. & Trofe, J. (2007) Posttransplant lymphoproliferative disorder. *Ann Pharmacother,* **41,** 1850-1858.

Feng, X., Hui, K.M., Younes, H.M. & Brickner, A.G. (2008) Targeting minor histocompatibility antigens in graft versus tumor or graft versus leukemia responses. *Trends Immunol,* **29,** 624-632.

Ferrand, C., Robinet, E., Contassot, E., Certoux, J.M., Lim, A., Herve, P. & Tiberghien, P. (2000) Retrovirus-mediated gene transfer in primary T lymphocytes: influence of the transduction/selection process and of ex vivo expansion on the T cell receptor beta chain hypervariable region repertoire. *Hum Gene Ther,* **11,** 1151-1164.

Feuchtinger, T., Richard, C., Joachim, S., Scheible, M.H., Schumm, M., Hamprecht, K., Martin, D., Jahn, G., Handgretinger, R. & Lang, P. (2008) Clinical grade generation of hexon-specific T cells for adoptive T-cell transfer as a treatment of adenovirus infection after allogeneic stem cell transplantation. *J Immunother,* **31,** 199-206.

Fischer, A., Blanche, S., Le Bidois, J., Bordigoni, P., Garnier, J.L., Niaudet, P., Morinet, F., Le Deist, F., Fischer, A.M., Griscelli, C. & et al. (1991) Anti-B-cell monoclonal antibodies in the treatment of severe B-cell lymphoproliferative syndrome following bone marrow and organ transplantation. *N Engl J Med,* **324,** 1451-1456.

Fleischhauer, K., Kernan, N.A., O'Reilly, R.J., Dupont, B. & Yang, S.Y. (1990) Bone marrow-allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. *N Engl J Med,* **323,** 1818-1822.

Foster, A.E., Dotti, G., Lu, A., Khalil, M., Brenner, M.K., Heslop, H.E., Rooney, C.M. & Bollard, C.M. (2008) Antitumor activity of EBV-specific T lymphocytes transduced with a dominant negative TGF-beta receptor. *J Immunother,* **31,** 500-505.

Fox, D.S., Cruz, M.C., Sia, R.A., Ke, H., Cox, G.M., Cardenas, M.E. & Heitman, J. (2001) Calcineurin regulatory subunit is essential for virulence and mediates interactions with FKBP12-FK506 in Cryptococcus neoformans. *Mol Microbiol,* **39,** 835-849.

Gattinoni, L., Klebanoff, C.A., Palmer, D.C., Wrzesinski, C., Kerstann, K., Yu, Z., Finkelstein, S.E., Theoret, M.R., Rosenberg, S.A. & Restifo, N.P. (2005) Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest,* **115,** 1616-1626.

Ghobrial, I.M., Habermann, T.M., Macon, W.R., Ristow, K.M., Larson, T.S., Walker, R.C., Ansell, S.M., Gores, G.J., Stegall, M.D. & McGregor, C.G. (2005) Differences between early and late posttransplant lymphoproliferative disorders in solid organ transplant patients: are they two different diseases? *Transplantation,* **79,** 244-247.

Goldman, J.P., Blundell, M.P., Lopes, L., Kinnon, C., Di Santo, J.P. & Thrasher, A.J. (1998) Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. *Br J Haematol,* **103,** 335-342.

Goulmy, E. (1996) Human minor histocompatibility antigens. *Curr Opin Immunol,* **8,** 75-81.

Goulmy, E., Schipper, R., Pool, J., Blokland, E., Falkenburg, J.H., Vossen, J., Gratwohl, A., Vogelsang, G.B., van Houwelingen, H.C. & van Rood, J.J. (1996) Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N Engl J Med,* **334,** 281-285.

Green, M. (2001) Management of Epstein-Barr virus-induced post-transplant lymphoproliferative disease in recipients of solid organ transplantation. *Am J Transplant,* **1,** 103-108.

Greenfield, H.M., Gharib, M.I., Turner, A.J., Guiver, M., Carr, T., Will, A.M. & Wynn, R.F. (2006) The impact of monitoring Epstein-Barr virus PCR in paediatric bone marrow transplant patients: can it successfully predict outcome and guide intervention? *Pediatr Blood Cancer,* **47,** 200-205.

Griffith, J.P., Kim, J.L., Kim, E.E., Sintchak, M.D., Thomson, J.A., Fitzgibbon, M.J., Fleming, M.A., Caron, P.R., Hsiao, K. & Navia, M.A. (1995) X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell,* **82,** 507-522.

Gross, T.G., Bucuvalas, J.C., Park, J.R., Greiner, T.C., Hinrich, S.H., Kaufman, S.S., Langnas, A.N., McDonald, R.A., Ryckman, F.C., Shaw, B.W., Sudan, D.L. & Lynch, J.C. (2005) Low-Dose Chemotherapy for Epstein-Barr Virus-Positive Post-Transplantation Lymphoproliferative Disease in Children After Solid Organ Transplantation. *J Clin Oncol,* **23,** 6481-6488.

Guppy, A.E., Rawlings, E., Madrigal, J.A., Amlot, P.L. & Barber, L.D. (2007) A quantitative assay for Epstein-Barr Virus-specific immunity shows interferon-gamma producing CD8+ T cells increase during immunosuppression reduction

to treat posttransplant lymphoproliferative disease. *Transplantation,* **84,** 1534-1539.

Gustafsson, A., Levitsky, V., Zou, J.Z., Frisan, T., Dalianis, T., Ljungman, P., Ringden, O., Winiarski, J., Ernberg, I. & Masucci, M.G. (2000) Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. *Blood,* **95,** 807-814.

Gutierrez-Dalmau, A. & Campistol, J.M. (2007) Immunosuppressive therapy and malignancy in organ transplant recipients: a systematic review. *Drugs,* **67,** 1167-1198.

Hacein-Bey-Abina, S., Garrigue, A., Wang, G.P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K., Asnafi, V., MacIntyre, E., Dal Cortivo, L., Radford, I., Brousse, N., Sigaux, F., Moshous, D., Hauer, J., Borkhardt, A., Belohradsky, B.H., Wintergerst, U., Velez, M.C., Leiva, L., Sorensen, R., Wulffraat, N., Blanche, S., Bushman, F.D., Fischer, A. & Cavazzana-Calvo, M. (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest,* **118,** 3132-3142.

Hacein-Bey-Abina, S., Kalle, C.V., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J.I., Basile, G.d.S., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L.E., Wissler, M., Prinz, C., Rabbitts, T.H., Deist, F.L., Fischer, A. & Cavazzana-Calvo, M. (2003) LMO2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1. *Science,* **302,** 415-419.

Hale, G.A., Pule, M., Amrolia, P., Weiss, H., Bollard, C., Rochester, R., Brenner, M., Rooney, C. & Heslop, H. (2008) Long-Term Follow-Up of Administration of Donor-Derived EBV-Specific CTLs to Prevent and Treat EBV Lymphoma After Hemopoietic Stem Cell Transplant. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation,* **14,** 3.

Hammerschmidt, W. & Sugden, B. (1989) Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. *Nature,* **340,** 393-397.

Haque, T., Amlot, P.L., Helling, N., Thomas, J.A., Sweny, P., Rolles, K., Burroughs, A.K., Prentice, H.G. & Crawford, D.H. (1998) Reconstitution of EBV-specific T cell immunity in solid organ transplant recipients. *J Immunol,* **160,** 6204-6209.

Haque, T., Wilkie, G.M., Jones, M.M., Higgins, C.D., Urquhart, G., Wingate, P., Burns, D., McAulay, K., Turner, M., Bellamy, C., Amlot, P.L., Kelly, D., MacGilchrist, A., Gandhi, M.K., Swerdlow, A.J. & Crawford, D.H. (2007) Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation

lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood,* **110,** 1123-1131.

Harris, N.L., Jaffe, E.S., Diebold, J., Flandrin, G., Muller-Hermelink, H.K., Vardiman, J., Lister, T.A. & Bloomfield, C.D. (2000) The World Health Organization classification of neoplastic diseases of the haematopoietic and lymphoid tissues: Report of the Clinical Advisory Committee Meeting, Airlie House, Virginia, November 1997. *Histopathology,* **36,** 69-86.

Hayashi, R.J., Kraus, M.D., Patel, A.L., Canter, C., Cohen, A.H., Hmiel, P., Howard, T., Huddleston, C., Lowell, J.A., Mallory, G., Jr., Mendeloff, E., Molleston, J., Sweet, S. & DeBaun, M.R. (2001) Posttransplant lymphoproliferative disease in children: correlation of histology to clinical behavior. *J Pediatr Hematol Oncol,* **23,** 14-18.

He, J.S. & Ostergaard, H.L. (2007) CTLs contain and use intracellular stores of FasL distinct from cytolytic granules. *J Immunol,* **179,** 2339-2348.

Heslop, H., Slobod, K.S., Pule, M., Hale, G.A., Rousseau, A., Smith, C.A., Bollard, C., Liu, H., Wu, J., Rochester, R., Amrolia, P., Hurwitz, J.L., Brenner, M. & Rooney, C. (2009) Long term outcome of EBV specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood,* **Submitted**.

Heslop, H.E., Brenner, M.K., Rooney, C., Krance, R.A., Roberts, W.M., Rochester, R., Smith, C.A., Turner, V., Sixbey, J., Moen, R. & et al. (1994a) Administration of neomycin-resistance-gene-marked EBV-specific cytotoxic T lymphocytes to recipients of mismatched-related or phenotypically similar unrelated donor marrow grafts. *Hum Gene Ther,* **5,** 381-397.

Heslop, H.E., Brenner, M.K. & Rooney, C.M. (1994b) Donor T cells to treat EBV-associated lymphoma. *N Engl J Med,* **331,** 679-680.

Heslop, H.E., Ng, C.Y., Li, C., Smith, C.A., Loftin, S.K., Krance, R.A., Brenner, M.K. & Rooney, C.M. (1996) Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med,* **2,** 551-555.

Hislop, A.D., Taylor, G.S., Sauce, D. & Rickinson, A.B. (2007) Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol,* **25,** 587-617.

Ho, M., Miller, G., Atchison, R.W., Breinig, M.K., Dummer, J.S., Andiman, W., Starzl, T.E., Eastman, R., Griffith, B.P., Hardesty, R.L. & et al. (1985) Epstein-Barr virus infections and DNA hybridization studies in posttransplantation lymphoma and lymphoproliferative lesions: the role of primary infection. *J Infect Dis,* **152,** 876-886.

Ho, S., Clipstone, N., Timmermann, L., Northrop, J., Graef, I., Fiorentino, D., Nourse, J. & Crabtree, G.R. (1996) The mechanism of action of cyclosporin A and FK506. *Clin Immunol Immunopathol,* **80,** S40-45.

Hopwood, P. & Crawford, D.H. (2000) The role of EBV in post-transplant malignancies: a review. *J Clin Pathol,* **53,** 248-254.

Howe, S.J. (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *The Journal of Clinical Investigation,* **118,** 3143-3150.

Howe, S.J., Mansour, M.R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempski, H., Brugman, M.H., Pike-Overzet, K., Chatters, S.J., de Ridder, D., Gilmour, K.C., Adams, S., Thornhill, S.I., Parsley, K.L., Staal, F.J., Gale, R.E., Linch, D.C., Bayford, J., Brown, L., Quaye, M., Kinnon, C., Ancliff, P., Webb, D.K., Schmidt, M., von Kalle, C., Gaspar, H.B. & Thrasher, A.J. (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest,* **118,** 3143-3150.

Hsieh, W.S., Lemas, M.V. & Ambinder, R.F. (1999) The biology of Epstein-Barr virus in post-transplant lymphoproliferative disease. *Transpl Infect Dis,* **1,** 204-212.

Huai, Q., Kim, H.Y., Liu, Y., Zhao, Y., Mondragon, A., Liu, J.O. & Ke, H. (2002) Crystal structure of calcineurin-cyclophilin-cyclosporin shows common but distinct recognition of immunophilin-drug complexes. *Proc Natl Acad Sci U S A,* **99,** 12037-12042.

Im, S.H. & Rao, A. (2004) Activation and deactivation of gene expression by Ca2+/calcineurin-NFAT-mediated signaling. *Mol Cells,* **18,** 1-9.

Issa, N., Amer, H., Dean, P.G., Kremers, W.K., Kudva, Y.C., Rostambeigi, N., Cosio, F.G., Larson, T.S., Habermann, T.M., Stegall, M.D. & Griffin, M.D. (2009) Posttransplant lymphoproliferative disorder following pancreas transplantation. *Am J Transplant,* **9,** 1894-1902.

Issa, N.C. & Fishman, J.A. (2009) Infectious complications of antilymphocyte therapies in solid organ transplantation. *Clin Infect Dis,* **48,** 772-786.

Jin, L. & Harrison, S.C. (2002) Crystal structure of human calcineurin complexed with cyclosporin A and human cyclophilin. *Proc Natl Acad Sci U S A,* **99,** 13522-13526.

Johannessen, I. (2002) Epstein-Barr virus, post-transplant lymphoproliferative disease and animal models. [Miscellaneous Article]. *Reviews in Medical Microbiology,* **13,** 129-140.

Karlsson, H., Brewin, J., Kinnon, C., Veys, P. & Amrolia, P.J. (2007) Generation of trispecific cytotoxic T cells recognizing cytomegalovirus, adenovirus, and

Epstein-Barr virus: an approach for adoptive immunotherapy of multiple pathogens. *J Immunother,* **30,** 544-556.

Kataoka, T. & Nagai, K. (2000) Involvement of FK506-sensitive and insensitive granule exocytosis pathways in perforin-dependent target cell lysis mediated by a CD8+ CTL clone. *Immunol Lett,* **72,** 49-52.

Kawamura, A. & Su, M.S. (1995) Interaction of FKBP12-FK506 with calcineurin A at the B subunit-binding domain. *J Biol Chem,* **270,** 15463-15466.

Ke, H. & Huai, Q. (2003) Structures of calcineurin and its complexes with immunophilins-immunosuppressants. *Biochem Biophys Res Commun,* **311,** 1095-1102.

Khanna, R., Bell, S., Sherritt, M., Galbraith, A., Burrows, S.R., Rafter, L., Clarke, B., Slaughter, R., Falk, M.C., Douglass, J., Williams, T., Elliott, S.L. & Moss, D.J. (1999) Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. *Proc Natl Acad Sci U S A,* **96,** 10391-10396.

Kiani, A., Garcia-Cozar, F.J., Habermann, I., Laforsch, S., Aebischer, T., Ehninger, G. & Rao, A. (2001) Regulation of interferon-gamma gene expression by nuclear factor of activated T cells. *Blood,* **98,** 1480-1488.

Kiani, A., Rao, A. & Aramburu, J. (2000) Manipulating immune responses with immunosuppressive agents that target NFAT. *Immunity,* **12,** 359-372.

Kieff, E. & Rickinson, A.B. (2007) Epstein-Barr Virus and its replication. In: *Field's Virology* (ed. by D.M. Knipe & P.M. Howley), Vol. 2, pp. 2603-2654. Lippincott Williams and Wilkins.

Kissinger, C.R., Parge, H.E., Knighton, D.R., Lewis, C.T., Pelletier, L.A., Tempczyk, A., Kalish, V.J., Tucker, K.D., Showalter, R.E., Moomaw, E.W. & et al. (1995) Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. *Nature,* **378,** 641-644.

Knight, J.S., Tsodikov, A., Cibrik, D.M., Ross, C.W., Kaminski, M.S. & Blayney, D.W. (2009) Lymphoma After Solid Organ Transplantation: Risk, Response to Therapy, and Survival at a Transplantation Center. *J Clin Oncol*.

Kuppers, R. (2003) B cells under influence: transformation of B cells by Epstein-Barr virus. *Nat Rev Immunol,* **3,** 801-812.

Lacerda, J.F., Ladanyi, M., Louie, D.C., Fernandez, J.M., Papadopoulos, E.B. & O'Reilly, R.J. (1996) Human Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes home preferentially to and induce selective regressions of autologous EBV-induced B cell lymphoproliferations in xenografted C.B-17 scid/scid mice. *J Exp Med,* **183,** 1215-1228.

Landewe, R.B., van den Borne, B.E., Breedveld, F.C. & Dijkmans, B.A. (1999) Does cyclosporin A cause cancer? *Nat Med,* **5,** 714.

Landgren, O., Gilbert, E.S., Rizzo, J.D., Socie, G., Banks, P.M., Sobocinski, K.A., Horowitz, M.M., Jaffe, E.S., Kingma, D.W., Travis, L.B., Flowers, M.E., Martin, P.J., Deeg, H.J. & Curtis, R.E. (2009) Risk factors for lymphoproliferative disorders after allogeneic hematopoietic cell transplantation. *Blood,* **113,** 4992-5001.

Leblond, V. & Choquet, S. (2004) Lymphoproliferative disorders after liver transplantation. *Journal of Hepatology,* **40,** 728-735.

Leblond, V., Davi, F., Charlotte, F., Dorent, R., Bitker, M.O., Sutton, L., Gandjbakhch, I., Binet, J.L. & Raphael, M. (1998) Posttransplant lymphoproliferative disorders not associated with Epstein-Barr virus: a distinct entity? *J Clin Oncol,* **16,** 2052-2059.

Lee, S.P., Brooks, J.M., Al-Jarrah, H., Thomas, W.A., Haigh, T.A., Taylor, G.S., Humme, S., Schepers, A., Hammerschmidt, W., Yates, J.L., Rickinson, A.B. & Blake, N.W. (2004) CD8 T Cell Recognition of Endogenously Expressed Epstein-Barr Virus Nuclear Antigen 1. *J. Exp. Med.,* **199,** 1409-1420.

Lee, T.C., Goss, J.A., Rooney, C.M., Heslop, H.E., Barshes, N.R., Caldwell, Y.M., Gee, A.P., Scott, J.D. & Savoldo, B. (2006a) Quantification of a low cellular immune response to aid in identification of pediatric liver transplant recipients at high-risk for EBV infection. *Clin Transplant,* **20,** 689-694.

Lee, T.C., Savoldo, B., Barshes, N.R., Rooney, C.M., Heslop, H.E., Gee, A.P., Caldwell, Y., Scott, J.D. & Goss, J.A. (2006b) Use of cytokine polymorphisms and Epstein-Barr virus viral load to predict development of post-transplant lymphoproliferative disorder in paediatric liver transplant recipients. *Clin Transplant,* **20,** 389-393.

Lee, T.C., Savoldo, B., Rooney, C.M., Heslop, H.E., Gee, A.P., Caldwell, Y., Barshes, N.R., Scott, J.D., Bristow, L.J., O'Mahony, C.A. & Goss, J.A. (2005) Quantitative EBV viral loads and immunosuppression alterations can decrease PTLD incidence in pediatric liver transplant recipients. *Am J Transplant,* **5,** 2222-2228.

Leen, A.M., Myers, G.D., Sili, U., Huls, M.H., Weiss, H., Leung, K.S., Carrum, G., Krance, R.A., Chang, C.C., Molldrem, J.J., Gee, A.P., Brenner, M.K., Heslop, H.E., Rooney, C.M. & Bollard, C.M. (2006) Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med,* **12,** 1160-1166.

Levine, P.H., Ablashi, D.V., Berard, C.W., Carbone, P.P., Waggoner, D.E. & Malan, L. (1971) Elevated antibody titers to Epstein-Barr virus in Hodgkin's disease. *Cancer,* **27,** 416-421.

Levitskaya, J., Coram, M., Levitsky, V., Imreh, S., Steigerwald-Mullen, P.M., Klein, G., Kurilla, M.G. & Masucci, M.G. (1995) Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature,* **375,** 685-688.

Lim, G.Y., Newman, B., Kurland, G. & Webber, S.A. (2002) Posttransplantation lymphoproliferative disorder: manifestations in pediatric thoracic organ recipients. *Radiology,* **222,** 699-708.

Lim, W.H., Russ, G.R. & Coates, P.T. (2006) Review of Epstein-Barr virus and post-transplant lymphoproliferative disorder post-solid organ transplantation. *Nephrology (Carlton),* **11,** 355-366.

Liu, D., Tammik, C., Zou, J.Z., Ernberg, I., Masucci, M.G., Ringden, O. & Levitsky, V. (2004) Effect of combined T- and B-cell depletion of allogeneic HLA-mismatched bone marrow graft on the magnitude and kinetics of Epstein-Barr virus load in the peripheral blood of bone marrow transplant recipients. *Clin Transplant,* **18,** 518-524.

Liu, J., Farmer, J.D., Jr., Lane, W.S., Friedman, J., Weissman, I. & Schreiber, S.L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell,* **66,** 807-815.

Lopes, C.T., Gallo, A.P., Palma, P.V., Cury, P.M. & Bueno, V. (2008) Skin allograft survival and analysis of renal parameters after FTY720 + tacrolimus treatment in mice. *Transplant Proc,* **40,** 856-860.

Lynch, B.A., Vasef, M.A., Comito, M., Gilman, A.L., Lee, N., Ritchie, J., Rumelhart, S., Holida, M. & Goldman, F. (2003) Effect of in vivo lymphocyte-depleting strategies on development of lymphoproliferative disorders in children post allogeneic bone marrow transplantation. *Bone Marrow Transplant,* **32,** 527-533.

Mackinnon, S., Thomson, K., Verfuerth, S., Peggs, K. & Lowdell, M. (2008) Adoptive cellular therapy for cytomegalovirus infection following allogeneic stem cell transplantation using virus-specific T cells. *Blood Cells Mol Dis,* **40,** 63-67.

Mancao, C., Altmann, M., Jungnickel, B. & Hammerschmidt, W. (2005) Rescue of "crippled" germinal center B cells from apoptosis by Epstein-Barr virus. *Blood,* **106,** 4339-4344.

Mancao, C. & Hammerschmidt, W. (2007) Epstein-Barr virus latent membrane protein 2A is a B-cell receptor mimic and essential for B-cell survival. *Blood,* **110,** 3715-3721.

Marijt, W.A.E., Heemskerk, M.H.M., Kloosterboer, F.M., Goulmy, E., Kester, M.G.D., van der Hoorn, M., van Luxemburg-Heys, S.A.P., Hoogeboom, M., Mutis, T., Drijfhout, J.W., van Rood, J.J., Willemze, R. & Falkenburg, J.H.F. (2003) Hematopoiesis-restricted minor histocompatibility antigens HA-1- or

HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proceedings of the National Academy of Sciences of the United States of America,* **100,** 2742-2747.

Markasz, L., Vanherberghen, B., Flaberg, E., Otvos, R., Stuber, G., Gustafsson Jernberg, A., Olah, E., Skribek, H. & Szekely, L. (2009) NK cell-mediated lysis is essential to kill Epstein-Barr virus transformed lymphoblastoid B cells when using rituximab. *Biomed Pharmacother,* **63,** 413-420.

Martelius, T., Lappalainen, M., Aalto, S.M., Nihtinen, A., Hedman, K. & Anttila, V.J. (2009) Clinical characteristics, outcome and the role of viral load in nontransplant patients with Epstein-Barr viraemia. *Clin Microbiol Infect*.

Matsuda, S., Shibasaki, F., Takehana, K., Mori, H., Nishida, E. & Koyasu, S. (2000) Two distinct action mechanisms of immunophilin-ligand complexes for the blockade of T-cell activation. *EMBO Rep,* **1,** 428-434.

McAlister, V.C., Keshavamurthy, M. & Lee, T.D. (1999) Oral delivery of liposomal tacrolimus: increased efficacy and reduced toxicity. *Transplant Proc,* **31,** 1110.

McAulay, K.A., Haque, T. & Crawford, D.H. (2009) Tumour necrosis factor gene polymorphism: a predictive factor for the development of post-transplant lymphoproliferative disease. *Br J Cancer,* **101,** 1019-1027.

McDiarmid, S.V., Jordan, S., Kim, G.S., Toyoda, M., Goss, J.A., Vargas, J.H., Martin, M.G., Bahar, R., Maxfield, A.L., Ament, M.E. & Busuttil, R.W. (1998) Prevention and preemptive therapy of postransplant lymphoproliferative disease in pediatric liver recipients. *Transplantation,* **66,** 1604-1611.

Medyouf, H., Alcalde, H., Berthier, C., Guillemin, M.C., dos Santos, N.R., Janin, A., Decaudin, D., de The, H. & Ghysdael, J. (2007) Targeting calcineurin activation as a therapeutic strategy for T-cell acute lymphoblastic leukemia. *Nat Med,* **13,** 736-741.

Meij, P., van Esser, J.W.J., Niesters, H.G.M., van Baarle, D., Miedema, F., Blake, N., Rickinson, A.B., Leiner, I., Pamer, E., Lowenberg, B., Cornelissen, J.J. & Gratama, J.W. (2003) Impaired recovery of Epstein-Barr virus (EBV)--specific CD8+ T lymphocytes after partially T-depleted allogeneic stem cell transplantation may identify patients at very high risk for progressive EBV reactivation and lymphoproliferative disease. *Blood,* **101,** 4290-4297.

Merlo, A., Turrini, R., Dolcetti, R., Zanovello, P., Amadori, A. & Rosato, A. (2008) Adoptive cell therapy against EBV-related malignancies: a survey of clinical results. *Expert Opin Biol Ther,* **8,** 1265-1294.

Messahel, B., Taj, M.M., Hobson, R., Hadzic, N., Ramsay, A., Hann, I. & Pinkerton, R. (2006) Single agent efficacy of rituximab in childhood immunosuppression related lymphoproliferative disease: a United Kingdom Children's Cancer

Study Group (UKCCSG) retrospective review. *Leuk Lymphoma,* **47,** 2584-2589.

Mielke, S., Nunes, R., Rezvani, K., Fellowes, V.S., Venne, A., Solomon, S.R., Fan, Y., Gostick, E., Price, D.A., Scotto, C., Read, E.J. & Barrett, A.J. (2008) A clinical-scale selective allodepletion approach for the treatment of HLA-mismatched and matched donor-recipient pairs using expanded T lymphocytes as antigen-presenting cells and a TH9402-based photodepletion technique. *Blood,* **111,** 4392-4402.

Milan, D., Griffith, J., Su, M., Price, E.R. & McKeon, F. (1994) The latch region of calcineurin B is involved in both immunosuppressant-immunophilin complex docking and phosphatase activation. *Cell,* **79,** 437-447.

Morgan, R.A., Dudley, M.E., Wunderlich, J.R., Hughes, M.S., Yang, J.C., Sherry, R.M., Royal, R.E., Topalian, S.L., Kammula, U.S., Restifo, N.P., Zheng, Z., Nahvi, A., de Vries, C.R., Rogers-Freezer, L.J., Mavroukakis, S.A. & Rosenberg, S.A. (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science,* **314,** 126-129.

Mueller, K., Schweier, O. & Pircher, H. (2008) Efficacy of IL-2- versus IL-15-stimulated CD8 T cells in adoptive immunotherapy. *Eur J Immunol,* **38,** 2874-2885.

Nagy, N., Matskova, L., Kis, L.L., Hellman, U., Klein, G. & Klein, E. (2009) The proapoptotic function of SAP provides a clue to the clinical picture of X-linked lymphoproliferative disease. *Proc Natl Acad Sci U S A,* **106,** 11966-11971.

Nalesnik, M.A. (1998) Clinical and pathological features of post-transplant lymphoproliferative disorders (PTLD). *Springer Semin Immunopathol,* **20,** 325-342.

Nash, A.A., Dutia, B.M., Stewart, J.P. & Davison, A.J. (2001) Natural history of murine gamma-herpesvirus infection. *Philos Trans R Soc Lond B Biol Sci,* **356,** 569-579.

Nelson, B.P., Nalesnik, M.A., Bahler, D.W., Locker, J., Fung, J.J. & Swerdlow, S.H. (2000) Epstein-Barr virus-negative post-transplant lymphoproliferative disorders: a distinct entity? *Am J Surg Pathol,* **24,** 375-385.

Nguyen, V.T., Morange, M. & Bensaude, O. (1988) Firefly luciferase luminescence assays using scintillation counters for quantitation in transfected mammalian cells. *Anal Biochem,* **171,** 404-408.

Omar, H., Hagglund, H., Gustafsson-Jernberg, A., Leblanc, K., Mattsson, J., Remberger, M., Ringden, O., Sparrelid, E., Sundin, M., Winiarski, J., Yun, Z. & Ljungman, P. (2009) Targeted monitoring of patients at high risk of post-transplant lymphoproliferative disease by quantitative Epstein-Barr virus polymerase chain reaction. *Transpl Infect Dis*.

Opelz, G. & Dohler, B. (2004) Lymphomas After Solid Organ Transplantation: A Collaborative Transplant Study Report. *American Journal of Transplantation,* **4,** 222-230.

Papadopoulos, E.B., Ladanyi, M., Emanuel, D., Mackinnon, S., Boulad, F., Carabasi, M.H., Castro-Malaspina, H., Childs, B.H., Gillio, A.P., Small, T.N. & et al. (1994) Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N Engl J Med,* **330,** 1185-1191.

Peggs, K.S., Verfuerth, S., Pizzey, A., Khan, N., Guiver, M., Moss, P.A. & Mackinnon, S. (2003) Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. *Lancet,* **362,** 1375-1377.

Penn, I. (1987) Cancers following cyclosporine therapy. *Transplant Proc,* **19,** 2211-2213.

Pinkerton, C.R., Hann, I., Weston, C.L., Mapp, T., Wotherspoon, A., Hobson, R., Kelly, D.A., Vergani, D., Hadzic, D., Rees, L., Burke, M. & Alero Thomas, J. (2002) Immunodeficiency-related lymphoproliferative disorders: prospective data from the United Kingdom Children's Cancer Study Group Registry. *Br J Haematol,* **118,** 456-461.

Preiksaitis, J.K. (2004) New developments in the diagnosis and management of posttransplantation lymphoproliferative disorders in solid organ transplant recipients. *Clin Infect Dis,* **39,** 1016-1023.

Pudney, V.A., Leese, A.M., Rickinson, A.B. & Hislop, A.D. (2005) CD8+ immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells. *J Exp Med,* **201,** 349-360.

Pule, M., Rosseau, A., Bollard, C.M., Hulse, H., Smith, S., Rochester, R., Benaim, E., Brenner, M.K., Rooney, C. & Heslop, H.E. (2004) Long-term safety and persistence data after infusion of retrovirally marked EBV-CTLs. *Blood,* **102,** 745a (abstract).

Pule, M.A., Savoldo, B., Myers, G.D., Rossig, C., Russell, H.V., Dotti, G., Huls, M.H., Liu, E., Gee, A.P., Mei, Z., Yvon, E., Weiss, H.L., Liu, H., Rooney, C.M., Heslop, H.E. & Brenner, M.K. (2008) Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med,* **14,** 1264-1270.

Quintarelli, C., Vera, J.F., Savoldo, B., Giordano Attianese, G.M.P., Pule, M., Foster, A.E., Heslop, H.E., Rooney, C.M., Brenner, M.K. & Dotti, G. (2007) Co-expression of cytokine and suicide genes to enhance the activity and safety of tumor-specific cytotoxic T lymphocytes. *Blood,* **110,** 2793-2802.

Quintas-Cardama, A., Yeh, R.K., Hollyman, D., Stefanski, J., Taylor, C., Nikhamin, Y., Imperato, G., Sadelain, M., RiviÃ¨re, I. & Brentjens, R.J. (2007) Multifactorial Optimization of Gammaretroviral Gene Transfer into Human T Lymphocytes for Clinical Application. *Human Gene Therapy,* **18,** 1253-1260.

Rabson, M., Gradoville, L., Heston, L. & Miller, G. (1982) Non-immortalizing P3J-HR-1 Epstein-Barr virus: a deletion mutant of its transforming parent, Jijoye. *J. Virol.,* **44,** 834-844.

Rapoport, A.P., Stadtmauer, E.A., Aqui, N., Badros, A., Cotte, J., Chrisley, L., Veloso, E., Zheng, Z., Westphal, S., Mair, R., Chi, N., Ratterree, B., Pochran, M.F., Natt, S., Hinkle, J., Sickles, C., Sohal, A., Ruehle, K., Lynch, C., Zhang, L., Porter, D.L., Luger, S., Guo, C., Fang, H.B., Blackwelder, W., Hankey, K., Mann, D., Edelman, R., Frasch, C., Levine, B.L., Cross, A. & June, C.H. (2005) Restoration of immunity in lymphopenic individuals with cancer by vaccination and adoptive T-cell transfer. *Nat Med,* **11,** 1230-1237.

Recchia, A., Bonini, C., Magnani, Z., Urbinati, F., Sartori, D., Muraro, S., Tagliafico, E., Bondanza, A., Stanghellini, M.T., Bernardi, M., Pescarollo, A., Ciceri, F., Bordignon, C. & Mavilio, F. (2006) Retroviral vector integration deregulates gene expression but has no consequence on the biology and function of transplanted T cells. *Proc Natl Acad Sci U S A,* **103,** 1457-1462.

Rechsteiner, M.P., Berger, C., Zauner, L., Sigrist, J.A., Weber, M., Longnecker, R., Bernasconi, M. & Nadal, D. (2008) Latent membrane protein 2B regulates susceptibility to induction of lytic Epstein-Barr virus infection. *J Virol,* **82,** 1739-1747.

Reddy, S., Piccione, D., Takita, H. & Bankert, R.B. (1987) Human lung tumor growth established in the lung and subcutaneous tissue of mice with severe combined immunodeficiency. *Cancer Res,* **47,** 2456-2460.

Rickinson, A.B. & Kieff, E. (2007) Epstein-Barr Virus. In: *Field's Virology* (ed. by D.M. Knipe & P.M. Howley), Vol. 2, pp. 2656-2701. Lippincott Williams and Wilkins.

Rickinson, A.B., Lee, S.P. & Steven, N.M. (1996) Cytotoxic T lymphocyte responses to Epstein-Barr virus. *Curr Opin Immunol,* **8,** 492-497.

Riddell, S.R., Watanabe, K.S., Goodrich, J.M., Li, C.R., Agha, M.E. & Greenberg, P.D. (1992) Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science,* **257,** 238-241.

Riviere, I., Brose, K. & Mulligan, R.C. (1995) Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. *Proc Natl Acad Sci U S A,* **92,** 6733-6737.

Rogers, A.M., Thilenius, A.R. & Russell, J.H. (1997) Cyclosporine-insensitive partial signaling and multiple roles of Ca2+ in Fas ligand-induced lysis. *J Immunol,* **159,** 3140-3147.

Rooney, C.M., Smith, C.A., Ng, C.Y., Loftin, S., Li, C., Krance, R.A., Brenner, M.K. & Heslop, H.E. (1995) Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet,* **345,** 9-13.

Rooney, C.M., Smith, C.A., Ng, C.Y., Loftin, S.K., Sixbey, J.W., Gan, Y., Srivastava, D.K., Bowman, L.C., Krance, R.A., Brenner, M.K. & Heslop, H.E. (1998) Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood,* **92,** 1549-1555.

Roskrow, M.A., Suzuki, N., Gan, Y., Sixbey, J.W., Ng, C.Y., Kimbrough, S., Hudson, M., Brenner, M.K., Heslop, H.E. & Rooney, C.M. (1998) Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's disease. *Blood,* **91,** 2925-2934.

Rossig, C. & Brenner, M.K. (2004) Genetic modification of T lymphocytes for adoptive immunotherapy. *Mol Ther,* **10,** 5-18.

Rowley, J., Monie, A., Hung, C.F. & Wu, T.C. (2009) Expression of IL-15RA or an IL-15/IL-15RA fusion on CD8+ T cells modifies adoptively transferred T-cell function in cis. *Eur J Immunol,* **39,** 491-506.

Savoldo, B., Cubbage, M.L., Durett, A.G., Goss, J., Huls, M.H., Liu, Z., Teresita, L., Gee, A.P., Ling, P.D., Brenner, M.K., Heslop, H.E. & Rooney, C.M. (2002) Generation of EBV-Specific CD4+ Cytotoxic T Cells from Virus Naive Individuals. *J Immunol,* **168,** 909-918.

Savoldo, B., Goss, J., Liu, Z., Huls, M.H., Doster, S., Gee, A.P., Brenner, M.K., Heslop, H.E. & Rooney, C.M. (2001) Generation of autologous Epstein-Barr virus-specific cytotoxic T cells for adoptive immunotherapy in solid organ transplant recipients. *Transplantation,* **72,** 1078-1086.

Savoldo, B., Goss, J.A., Hammer, M.M., Zhang, L., Lopez, T., Gee, A.P., Lin, Y.F., Quiros-Tejeira, R.E., Reinke, P., Schubert, S., Gottschalk, S., Finegold, M.J., Brenner, M.K., Rooney, C.M. & Heslop, H.E. (2006) Treatment of solid organ transplant recipients with autologous Epstein Barr virus-specific cytotoxic T lymphocytes (CTLs). *Blood,* **108,** 2942-2949.

Savoldo, B., Rooney, C.M., Di Stasi, A., Abken, H., Hombach, A., Foster, A.E., Zhang, L., Heslop, H.E., Brenner, M.K. & Dotti, G. (2007) Epstein Barr virus specific cytotoxic T lymphocytes expressing the anti-CD30zeta artificial chimeric T-cell receptor for immunotherapy of Hodgkin disease. *Blood,* **110,** 2620-2630.

Schambach, A., Galla, M., Maetzig, T., Loew, R. & Baum, C. (2007) Improving transcriptional termination of self-inactivating gamma-retroviral and lentiviral vectors. *Mol Ther,* **15,** 1167-1173.

Sebelin-Wulf, K., Nguyen, T.D., Oertel, S., Papp-Vary, M., Trappe, R.U., Schulzki, A., Pezzutto, A., Riess, H. & Subklewe, M. (2007) Quantitative analysis of EBV-specific CD4/CD8 T cell numbers, absolute CD4/CD8 T cell numbers and EBV load in solid organ transplant recipients with PLTD. *Transpl Immunol,* **17,** 203-210.

Shahinian, V.B., Muirhead, N., Jevnikar, A.M., Leckie, S.H., Khakhar, A.K., Luke, P.P., Rizkalla, K.S., Hollomby, D.J. & House, A.A. (2003) Epstein-Barr virus seronegativity is a risk factor for late-onset posttransplant lymphoroliferative disorder in adult renal allograft recipients. *Transplantation,* **75,** 851-856.

Sherritt, M.A., Bharadwaj, M., Burrows, J.M., Morrison, L.E., Elliott, S.L., Davis, J.E., Kear, L.M., Slaughter, R.E., Bell, S.C., Galbraith, A.J., Khanna, R. & Moss, D.J. (2003) Reconstitution of the latent T-lymphocyte response to Epstein-Barr virus is coincident with long-term recovery from posttransplant lymphoma after adoptive immunotherapy. *Transplantation,* **75,** 1556-1560.

Shiba, N., Chan, M.C., Kwok, B.W., Valantine, H.A., Robbins, R.C. & Hunt, S.A. (2004) Analysis of survivors more than 10 years after heart transplantation in the cyclosporine era: Stanford experience. *J Heart Lung Transplant,* **23,** 155-164.

Smets, F., Latinne, D., Bazin, H., Reding, R., Otte, J.B., Buts, J.P. & Sokal, E.M. (2002) Ratio between Epstein-Barr viral load and anti-Epstein-Barr virus specific T-cell response as a predictive marker of posttransplant lymphoproliferative disease. *Transplantation,* **73,** 1603-1610.

Smith-Garvin, J.E., Koretzky, G.A. & Jordan, M.S. (2009) T cell activation. *Annu Rev Immunol,* **27,** 591-619.

Snow, A.L. & Martinez, O.M. (2007) Epstein-Barr virus: evasive maneuvers in the development of PTLD. *Am J Transplant,* **7,** 271-277.

Sokal, E.M., Antunes, H., Beguin, C., Bodeus, M., Wallemacq, P., de Ville de Goyet, J., Reding, R., Janssen, M., Buts, J.P. & Otte, J.B. (1997) Early signs and risk factors for the increased incidence of Epstein-Barr virus-related posttransplant lymphoproliferative diseases in pediatric liver transplant recipients treated with tacrolimus. *Transplantation,* **64,** 1438-1442.

Stauss, H.J., Thomas, S., Cesco-Gaspere, M., Hart, D.P., Xue, S.A., Holler, A., King, J., Wright, G., Perro, M., Pospori, C. & Morris, E. (2008) WT1-specific T cell receptor gene therapy: improving TCR function in transduced T cells. *Blood Cells Mol Dis,* **40,** 113-116.

Straathof, K.C., Pule, M.A., Yotnda, P., Dotti, G., Vanin, E.F., Brenner, M.K., Heslop, H.E., Spencer, D.M. & Rooney, C.M. (2005) An inducible caspase 9 safety switch for T-cell therapy. *Blood,* **105,** 4247-4254.

Sunil-Chandra, N.P., Arno, J., Fazakerley, J. & Nash, A.A. (1994) Lymphoproliferative disease in mice infected with murine gammaherpesvirus 68. *Am J Pathol,* **145,** 818-826.

Svoboda, J., Kotloff, R. & Tsai, D.E. (2006) Management of patients with post-transplant lymphoproliferative disorder: the role of rituximab. *Transpl Int,* **19,** 259-269.

Swerdlow, A.J., Higgins, C.D., Hunt, B.J., Thomas, J.A., Burke, M.M., Crawford, D.H. & Yacoub, M.H. (2000) Risk of lymphoid neoplasia after cardiothoracic transplantation. a cohort study of the relation to Epstein-Barr virus. *Transplantation,* **69,** 897-904.

Tanner, J.E. & Alfieri, C. (2001) The Epstein-Barr virus and post-transplant lymphoproliferative disease: interplay of immunosuppression, EBV, and the immune system in disease pathogenesis. *Transplant Infectious Disease,* **3,** 60-69.

Taylor, A.L., Marcus, R. & Bradley, J.A. (2005) Post-transplant lymphoproliferative disorders (PTLD) after solid organ transplantation. *Crit Rev Oncol Hematol,* **56,** 155-167.

Tellam, J., Connolly, G., Green, K.J., Miles, J.J., Moss, D.J., Burrows, S.R. & Khanna, R. (2004) Endogenous Presentation of CD8+ T Cell Epitopes from Epstein-Barr Virus-encoded Nuclear Antigen 1. *J. Exp. Med.,* **199,** 1421-1431.

Thompson, J.S. (1999) Intestinal transplantation. Experience in the United States. *Eur J Pediatr Surg,* **9,** 271-273.

Thorley-Lawson, D.A. & Allday, M.J. (2008) The curious case of the tumour virus: 50 years of Burkitt's lymphoma. *Nat Rev Microbiol,* **6,** 913-924.

Thornhill, S.I., Schambach, A., Howe, S.J., Ulaganathan, M., Grassman, E., Williams, D., Schiedlmeier, B., Sebire, N.J., Gaspar, H.B., Kinnon, C., Baum, C. & Thrasher, A.J. (2008) Self-inactivating gammaretroviral vectors for gene therapy of X-linked severe combined immunodeficiency. *Mol Ther,* **16,** 590-598.

Tiberghien, P., Ferrand, C., Lioure, B., Milpied, N., Angonin, R., Deconinck, E., Certoux, J.-M., Robinet, E., Saas, P., Petracca, B., Juttner, C., Reynolds, C.W., Longo, D.L., Herve, P. & Cahn, J.-Y. (2001) Administration of herpes simplex-thymidine kinase-expressing donor T cells with a T-cell-depleted allogeneic marrow graft. *Blood,* **97,** 63-72.

Traversari, C., Marktel, S., Magnani, Z., Mangia, P., Russo, V., Ciceri, F., Bonini, C. & Bordignon, C. (2007) The potential immunogenicity of the TK suicide gene

does not prevent full clinical benefit associated with the use of TK-transduced donor lymphocytes in HSCT for hematologic malignancies. *Blood,* **109,** 4708-4715.

Tsai, D.E., Hardy, C.L., Tomaszewski, J.E., Kotloff, R.M., Oltoff, K.M., Somer, B.G., Schuster, S.J., Porter, D.L., Montone, K.T. & Stadtmauer, E.A. (2001) Reduction in immunosuppression as initial therapy for posttransplant lymphoproliferative disorder: analysis of prognostic variables and long-term follow-up of 42 adult patients. *Transplantation,* **71,** 1076-1088.

Tsao, L. & Hsi, E.D. (2007) The clinicopathologic spectrum of posttransplantation lymphoproliferative disorders. *Arch Pathol Lab Med,* **131,** 1209-1218.

Uckert, W., Becker, C., Gladow, M., Klein, D., Kammertoens, T., Pedersen, L. & Blankenstein, T. (2000) Efficient gene transfer into primary human CD8+ T lymphocytes by MuLV-10A1 retrovirus pseudotype. *Hum Gene Ther,* **11,** 1005-1014.

Vaessen, L.M., Baan, C.C., Ouwehand, A.J., Balk, A.H., Jutte, N.H., Mochtar, B., Claas, F.H. & Weimar, W. (1994) Differential avidity and cyclosporine sensitivity of committed donor-specific graft-infiltrating cytotoxic T cells and their precursors. Relevance for clinical cardiac graft rejection. *Transplantation,* **57,** 1051-1059.

van Gorp, J., Doornewaard, H., Verdonck, L.F., Klöpping, C., Vos, P.F. & van den Tweel, J.G. (1994) Posttransplant t-cell lymphoma. Report of three cases and a review of the literature. *Cancer,* **73,** 3064-3072.

van Rhee, F. & Kolb, H.J. (1995) Donor leukocyte transfusions for leukemic relapse. *Curr Opin Hematol,* **2,** 423-430.

Vera, J.F., Brenner, L., Leen, A.M., Heslop, H.E., Dotti, G., Wilson, J. & Rooney, C.M. (2008) Rapid Generation of Antigen-Specific T Cells for Pre-Clinical and Clinical Applications Using a Novel Mini Cell Bioreactor. *ASH Annual Meeting Abstracts,* **112,** 208-.

Verschuuren, E.A., Stevens, S.J., van Imhoff, G.W., Middeldorp, J.M., de Boer, C., Koeter, G., The, T.H. & van Der Bij, W. (2002) Treatment of posttransplant lymphoproliferative disease with rituximab: the remission, the relapse, and the complication. *Transplantation,* **73,** 100-104.

Voo, K.S., Fu, T., Wang, H.Y., Tellam, J., Heslop, H.E., Brenner, M.K., Rooney, C.M. & Wang, R.-F. (2004) Evidence for the Presentation of Major Histocompatibility Complex Class I-restricted Epstein-Barr Virus Nuclear Antigen 1 Peptides to CD8+ T Lymphocytes. *J. Exp. Med.,* **199,** 459-470.

Wagar, E.J., Cromwell, M.A., Shultz, L.D., Woda, B.A., Sullivan, J.L., Hesselton, R.M. & Greiner, D.L. (2000) Regulation of human cell engraftment and

development of EBV-related lymphoproliferative disorders in Hu-PBL-scid mice. *J Immunol,* **165,** 518-527.

Wagner, H.J., Cheng, Y.C., Huls, M.H., Gee, A.P., Kuehnle, I., Krance, R.A., Brenner, M.K., Rooney, C.M. & Heslop, H.E. (2004) Prompt versus preemptive intervention for EBV lymphoproliferative disease. *Blood,* **103,** 3979-3981.

Walker, R.C., Paya, C.V., Marshall, W.F., Strickler, J.G., Wiesner, R.H., Velosa, J.A., Habermann, T.M., Daly, R.C. & McGregor, C.G. (1995) Pretransplantation seronegative Epstein-Barr virus status is the primary risk factor for posttransplantation lymphoproliferative disorder in adult heart, lung, and other solid organ transplantations. *J Heart Lung Transplant,* **14,** 214-221.

Walter, E.A., Greenberg, P.D., Gilbert, M.J., Finch, R.J., Watanabe, K.S., Thomas, E.D. & Riddell, S.R. (1995) Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med,* **333,** 1038-1044.

Wang, F., Gregory, C., Sample, C., Rowe, M., Liebowitz, D., Murray, R., Rickinson, A. & Kieff, E. (1990) Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *J Virol,* **64,** 2309-2318.

Webber, S.A., Naftel, D.C., Fricker, F.J., Olesnevich, P., Blume, E.D., Addonizio, L., Kirklin, J.K. & Canter, C.E. (2006) Lymphoproliferative disorders after paediatric heart transplantation: a multi-institutional study. *Lancet,* **367,** 233-239.

Wilkie, G.M., Taylor, C., Jones, M.M., Burns, D.M., Turner, M., Kilpatrick, D., Amlot, P.L., Crawford, D.H. & Haque, T. (2004) Establishment and Characterization of a Bank of Cytotoxic T Lymphocytes for Immunotherapy of Epstein-Barr Virus-Associated Diseases. *Journal of Immunotherapy,* **27,** 309-316.

Xue, S.A., Gao, L., Hart, D., Gillmore, R., Qasim, W., Thrasher, A., Apperley, J., Engels, B., Uckert, W., Morris, E. & Stauss, H. (2005) Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. *Blood,* **106,** 3062-3067.

Xue, S.A., Gao, L., Thomas, S., Hart, D.P., Xue, J.Z., Gillmore, R., Voss, R.H., Morris, E. & Stauss, H.J. (2009) Development of a WT1-TCR for clinical trials: engineered patient T cells can eliminate autologous leukemia blasts in NOD/SCID mice. *Haematologica*.

Yam, P., Jensen, M., Akkina, R., Anderson, J., Villacres, M.C., Wu, J., Zaia, J.A. & Yee, J.K. (2006) Ex vivo selection and expansion of cells based on expression of a mutated inosine monophosphate dehydrogenase 2 after HIV vector

transduction: effects on lymphocytes, monocytes, and CD34+ stem cells. *Mol Ther,* **14,** 236-244.

Yang, H., McAlister, V.C., Al-Jazaeri, A. & Wright, J.R., Jr. (2002) Liposomal encapsulation significantly enchances the immunosuppressive effect of tacrolimus in a discordant islet xenotransplant model. *Transplantation,* **73,** 710-713.

Yin, Y., Manoury, B. & Fahraeus, R. (2003) Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1. *Science,* **301,** 1371-1374.

Young, L.S. & Murray, P.G. (2003) Epstein-Barr virus and oncogenesis: from latent genes to tumours. *Oncogene,* **22,** 5108-5121.

Zaldumbide, A. & Hoeben, R.C. (2007) How not to be seen: immune-evasion strategies in gene therapy. *Gene Ther,* **15,** 239-246.

Zhan, X., Brown, B., Slobod, K.S. & Hurwitz, J.L. (2003) Inhibition of ex vivo-expanded cytotoxic T-lymphocyte function by high-dose cyclosporine. *Transplantation,* **76,** 739-740.

Zhou, X., Hu, W. & Qin, X. (2008) The Role of Complement in the Mechanism of Action of Rituximab for B-Cell Lymphoma: Implications for Therapy. *Oncologist,* **13,** 954-966.

Zhu, D., Cardenas, M.E. & Heitman, J. (1996) Calcineurin mutants render T lymphocytes resistant to cyclosporin A. *Mol Pharmacol,* **50,** 506-511.

Appendix 1

GENE THERAPY

Generation of EBV-specific cytotoxic T cells that are resistant to calcineurin inhibitors for the treatment of posttransplantation lymphoproliferative disease

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Epstein-Barr virus (EBV)–driven posttransplantation lymphoproliferative disease (PTLD) is a serious complication of immunosuppression after either stem cell transplantation (SCT) or solid organ transplantation (SOT). Adoptive transfer of EBV-specific cytotoxic T lymphocytes (EBV-CTLs) is an effective prophylaxis and treatment for PTLD after SCT, but not for PTLD after SOT when pharmacologic immunosuppression cannot be discontinued. We report the generation of calcineurin (CN) mutants that render EBV-CTL resistant to the immunosuppressants tacrolimus (FK506) and cyclosporin A (CsA): mutant CNa12 confers resistance to CsA but not FK506, and mutant CNa22 confers resistance to FK506 but not CsA, whereas mutant CNb30 renders CTLs resistant to both calcineurin inhibitors. Untransduced EBV-CTLs do not proliferate in the presence of FK506/CsA. However, EBV-CTLs transduced with a retroviral vector coding for these mutants retain the ability to both proliferate and secrete normal levels of interferon-γ in the presence therapeutic levels of FK506 (CNa12), CsA (CNa22), or both (CNb30). The cytotoxicity and phenotype of EBV-CTL lines were unaffected by expression of these mutant CNs. This approach should allow effective immunotherapy with EBV-CTLs in the SOT setting without risking the graft by reduction in immunosuppression, and represents a generic approach to improving immunotherapy in the face of immunosuppression. (Blood. 2009; 114:4792-4803)

Introduction

Posttransplantation lymphoproliferative disease (PTLD) is a serious complication of immunosuppression after either stem cell transplantation (SCT) or solid organ transplantation (SOT) and is associated with significant mortality.[1-5] B-cell proliferation leading to the development of PTLD is driven by Epstein-Barr virus (EBV), a ubiquitous γ herpesvirus which establishes latency in a pool of memory B cells after primary infection.[6,7] In immunocompetent individuals, outgrowth of EBV-infected B cells is prevented by virus-specific cytotoxic T lymphocytes (EBV-CTLs).[8] The lack of such an immune response early after T cell–depleted SCT, or as a result of the lifelong immune suppression necessary to prevent graft rejection after SOT, can result in uncontrolled EBV-driven B-cell proliferation and the development of PTLD.[7,9]

Because loss of cellular immunity to EBV plays a critical role in the pathogenesis of PTLD, adoptive immunotherapy to restore viral-specific immunity represents a logical approach to preventing this complication. In SCT, EBV-CTL lines generated ex vivo from the stem cell donor and infused prophylactically can prevent the development of PTLD in high-risk patients.[10-12] Furthermore, these donor EBV-CTLs have also been shown to induce durable responses in patients with established PTLD.[10,13] In these studies, immunosuppression was withdrawn or tapered before adoptive transfer of CTLs.

In contrast, in the SOT setting where immunosuppression could not be stopped, the use of EBV-CTLs has met with more limited success. Autologous EBV-CTLs generated from the organ recipient induce a transient increase in circulating EBV-CTL frequency, but variable reduction in the viral load and limited long-term clinical efficacy.[14,15] A clinical response was observed in one patient despite therapeutic doses of cyclosporin A (CsA); however, the patient relapsed 10 weeks later.[16] In addition, partly HLA-matched third-party EBV-CTLs induced clinical responses in 17 of 28 SOT patients with established life-threatening PTLD. However, it was notable that all patients in this study had a reduction of immunosuppression; 26 patients' suppressions stopped completely before EBV-CTL infusion.[17] These results demonstrate the potential clinical utility of EBV-CTLs infused after SOT; however, the success of immunotherapy seen in PTLD after SCT has not been matched thus far.

It is likely that the function of the infused EBV-CTLs is impaired by ongoing immunosuppression after SOT. Indeed, the major immunosuppressants used in this setting, tacrolimus (FK506) and CsA, have been shown to inhibit both the proliferation and function of EBV-CTLs in vitro.[18-20] Although reduction in immunosuppression is frequently used to restore some EBV-specific immunity in SOT patients with PTLD,[21,22] this must be balanced with the concurrent increased risk of graft rejection. For instance, in one major series, death from graft loss was as common as death from PTLD.[4] To address this, we investigated the possibility of engineering ex vivo–generated EBV-CTLs to be resistant to calcineurin inhibitors. We anticipate that these resistant CTLs could function effectively to treat PTLD in SOT patients without requiring a reduction in immunosuppression.

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We focused on the interactions between calcineurin (CN) and FK506/CsA, bound by their carrier proteins FKBP12/cyclopilin A (CyPA). CN is a heterodimeric serine-threonine phosphatase which is central to T-cell activation. After engagement of the T-cell receptor, CN dephosphorylates the transcription factor NFAT, allowing it to translocate to the nucleus and activate key target genes such as *IL2*.[23] FK506 in complex with FKBP12, or CsA in complex with CyPA, block NFAT access to CN′s active site, preventing its dephosphorylation, and thereby inhibiting T-cell activation.[24,25] We modified CN at key amino acid residues to disrupt docking of either or both FK506-FKBP12 and CsA-CyPA to produce CN mutants resistant to FK506 and/or CsA.[26,27] In a parallel approach, we mutated FKBP12 to disrupt its interaction with CN, thereby sequestering free FK506 in complex with mutant FKBP12. We anticipated that expression of such mutants in EBV-CTLs would render them resistant to immunosuppression.

Here, we demonstrate that EBV-CTLs can indeed be rendered resistant to the CN inhibitors CsA or FK506 by genetic engineering. This approach may allow EBV-CTLs to retain their function in immunosuppressed patients, allowing effective adoptive immunotherapy of PTLD while maintaining therapeutic immunosuppression.

Methods

Design and generation of CN and FKBP12 mutants

Crystallographic data were used to identify critical residues involved in the interaction between CN and the FK506/FKBP12 or CsA/CyPA heterodimers.[26-29] These were substituted with alternatives predicted to interrupt binding based on their charge and side-chain characteristics. Mutations were generated using overlap extension polymerase chain reaction (PCR) as published previously.[30] In brief, 2 fragments of CN or FKBP12 genes adjacent to the target site were amplified in initial mutagenesis PCRs using one complimentary external primer and one internal primer containing the desired sequence change. A total of 35 cycles of amplification were performed (40 seconds at 98°C, 30 seconds at 64°C, and 90 seconds at 72°C) using Phusion polymerase (NEB). Products were purifed using PCR clean-up columns (QIAGEN) and joined in a fusion PCR using the 2 external primers and amplification conditions described. CN and FKBP12 mutants were cloned as *MluI-NotI* fragments into the SFG-eGFP retroviral vector. This vector consists of Moloney murine leukemia virus (MMLV) long-terminal repeats LTRs driving transgene expression from the start codon of the deleted viral *env* gene, followed by an internal ribosomal entry site and an eGFP reporter gene.[31] Codon optimization was performed in-house. First, we designing an optimal sequence based on weighted optimization of codon usage for *Homo sapiens*, raising GC content to 70%, reduction of local hairpins and literal repeats to a minimum, and avoidance of splice signals. Next, the entire sequence was assembled with ligation-by-PCR using Phusion polymerase from overlapping oligonucleotides (IDT DNA). Finally, the synthetic DNA was directly cloned into the retroviral vector and confirmed to be correct by capillary sequencing (ABI 3130xl Genetic Analyzer; Applied Biosystems). The sequences of the CN genes wtCNa, CNa12, CNa22, wtCNb and CNb30 have been published in GenBank, with accession numbers GQ463593-GQ463597 and are available from AddGene with reference numbers 22489-22493.

Dual luciferase assay

293T cells were plated at 7×10^4 per well in 24-well tissue-culture plates (24 wp) in Dulbecco modified Eagle medium (DMEM; Gibco, Invitrogen) containing 10% fetal bovine serum (Sigma-Aldrich) without antibiotics. The following day, cells were triple-transfected with 400 ng of NFAT-RE_FLuc NFAT–responsive Firefly luciferase plasmid (Clontech), 8 ng of phRG-TK constitutively expressed *Renilla* luciferase control plasmid (Promega), and 600 ng of SFG-CN plasmid using Lipofectamine 2000 (Invitrogen). Two days later, cultures were stimulated with 20 ng/mL PMA and 1 μg/mL ionomycin (both Sigma-Aldrich) with or without 10 ng/mL FK506 (Astellas Pharma) or 200 ng/mL CsA (Novartis Pharmaceuticals). The next day, a dual luciferase assay was performed according to the manufacturer's instructions (Promega) and analyzed using a FLUOstar Optima Luminometer (BMG Labtech). Firefly luciferase activity was normalized to *Renilla* luciferase activity to control for transfection efficiency. Results were expressed as the percentage of activity of the stimulated sample for each construct without FK506/CsA (percentage of resistance).

Generation of retrovirus

For Jurkat transductions, RD114 pseudotyped transient retroviral supes were generated by triple transfection of 4.69 μg of Peq-Pam plasmid (Moloney GagPol), 3.125 μg of RDF plasmid (RD114 envelope[32]), and 4.69 μg of SFG-CN or SFG-FKBP12 plasmids into 293T cells using GeneJuice (Novagen). Supernatant was harvested after 48 and 72 hours. High-titer producer lines were generated by multiple transduction of FLY-RD18 cells[33] with GALV pseudotyped SFG retrovirus containing CN mutants, produced as described. FLY-RD18 cells were transduced daily for 2 weeks, bulk fluorescence-activated cell sorter (FACS)–sorted for high expression of eGFP, and single-cell cloned by limiting dilution; supernatant from the expanded clones was titred on 293T cells to identify the highest titer producers. These stable producer lines were used to generate high-titer retroviral supernatants for transduction of EBV-CTLs.

Transduction of Jurkat cells and EBV-CTLs

Non–tissue-culture–treated 24 wp were coated for 2 hours at 37°C with 7 μg/mL Retronectin (TaKaRa, Bio USA) in phosphate-buffered saline (PBS), washed once with PBS, and coated with 0.5 mL retrovirus for 30 minutes at room temperature. A total of 0.5×10^6 Jurkat cells or CTLs per well were transduced with 2 mL of retroviral supernatant (100 IU/mL IL-2 was added to EBV-CTLs), spun for 40 minutes at 1000*g*, and incubated at 37°C. EBV-CTLs were transduced 3 days after their second stimulation. Transduction efficiency was determined by expression of eGFP at 3 days for Jurkat cells and 7 days for EBV-CTLs.

Stimulation of Jurkat cell line and measurement of IL-2

Jurkat cells cultured in RPMI (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin were split 1:10 on day −1. For stimulation, 5×10^5 Jurkat cells were plated per well of a 48-well plate with 20 ng/mL PMA and 1 μg/mL ionomycin. FK506/CsA were added at increasing concentrations (0.5-40 ng/mL and 50-800 ng/mL, respectively). Cells were incubated for 24 hours, and samples of supernatant were frozen at −20°C for enzyme-linked immunosorbent assay (ELISA). To measure IL-2 secretion, the IL-2 DuoSet ELISA was used according to manufacturer's instructions (R&D Systems) and read using a FLUOstar Optima.

Generation of EBV-CTL lines

EBV-CTLs were generated as previously published.[18] In brief, EBV-CTLs were cultured in 45% Click medium (Irvine Scientific), 45% RPMI, and 10% FBS (both from HyClone) supplemented with 20 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. A total of 2×10^6 peripheral blood mononuclear cells (PBMCs) were plated per well of a 24-well plate and stimulated with 5×10^4 irradiated (40 Gy) autologous lymphoblastoid cell lines (LCLs). After 9 to 11 days, 10^6 viable cells per well were replated into 24-well plates and stimulated with 2.5×10^5 autologous irradiated LCLs. CTLs were restimulated in this way weekly thereafter. A total of 100 U/mL IL-2 (GenScript) was added on days 13 to 14 and changed at every stimulation and twice weekly.

Phenotyping EBV-CTL lines

Four days after the fifth stimulation with autologous LCLs, CTL lines were assessed for expression of surface markers using the following monoclonal antibodies: CD3, CD4, CD19, CD25 (Becton Dickinson), CD45RO (AbD Serotec), CD8, CD16, CD56, and CD62L (eBioscience). Appropriately

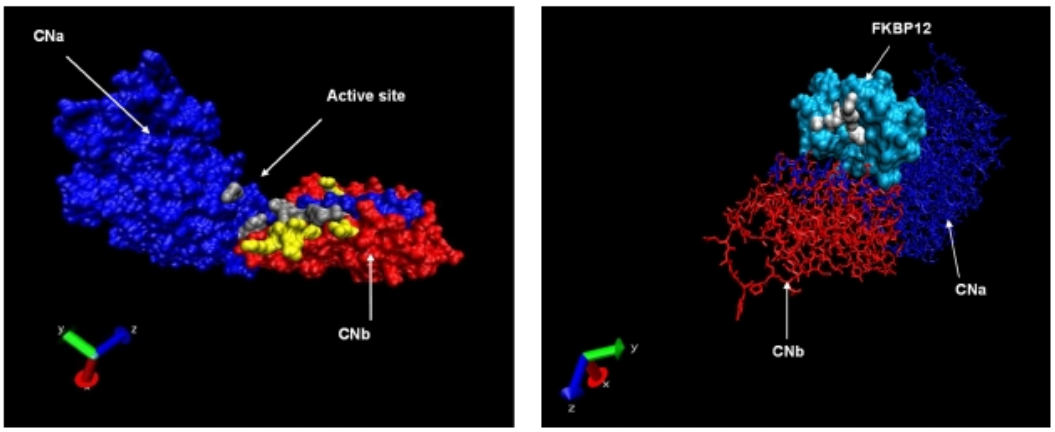

Figure 1. Location of mutated residues. (A) Crystallographic representation of the heterodimer of CNa (blue chain) and CNb (red chain). Mutated residues of CNa and CNb are shown in gray and yellow, respectively. (B) Reverse projection crystallographic representation of heterodimer of FKBP12 and FK506. CNa/b chains are included for orientation. Mutated residues of FKBP12 are shown in white.

matched isotype controls were included. Cells were analyzed on an LSRII flow cytometer (Becton Dickinson).

Antigen dependence

To confirm that transduced EBV-CTLs remained antigen dependent, after 4 stimulations with autologous LCLs, CTLs were cultured without further stimulation and cell growth was monitored thereafter. Some cells continued to receive 100 U/mL IL-2. Cells were counted weekly and replated at 10^6 viable cells per well of a 24-well plate.

^{3}H-thymidine proliferation assays

On the day of stimulation 6, 10^5 CTLs were plated in triplicate in U-bottomed 96-well plates, with or without stimulation by 2.5×10^4 autologous irradiated LCLs, in the presence or absence of 10 ng/mL FK506 or 200 ng/mL CsA. After 4 days, wells were pulsed with 1 μL (37 Bq) of ^{3}H-thymidine (Perkin Elmer) and incubated for a further 19 to 20 hours, when cells were harvested to a filter mat using a Tomtec MachIIIM Harvester 96. MeltiLex wax scintillation was applied (Perkin Elmer), and thymidine incorporation was measured using a Wallac 1450 MicroBeta trilux beta-counter (Perkin Elmer). Specific proliferation was calculated by subtracting mean counts per minute (cpm) of CTL- and LCL-alone control wells from those containing CTLs stimulated with LCLs.

Interferon-γ ELISA

At 24 hours after their sixth stimulation, samples of supernatant were taken from EBV-CTL cultures and frozen at −20°C. Interferon-γ (IFN-γ) concentration was measured using the IFN-γ Duo Set ELISA kit (R&D Systems).

^{51}Cr release cytotoxicity assays

Cytotoxicity of EBV-CTLs was determined using a standard chromium (^{51}Cr) release assay, as previously described.[10] In brief, 2×10^6 target cells (autologous/allogeneic LCLs) were incubated at 37°C with 37 MBq (1 mCi) ^{51}Cr-labeled sodium chromate (Perkin Elmer) for 1 hour and washed, and 5×10^3 targets were cocultured with EBV-CTLs in triplicate in V-bottomed 96-well plates at effector-target ratios of 30:1, 15:1, and 7.5:1 with or without 10 ng/mL FK506 or 200 ng/mL CsA. Target cells in complete media or lysed with 1% TritonX-100 determined spontaneous and maximum release, respectively. Supernatant was harvested after 4 to 6 hours, mixed with scintillation fluid (OptiPhase Supermix; Perkin Elmer) and read using a Wallac 1450 MicroBeta trilux. Percentage of specific lysis was calculated as follows: (specific release − spontaneous release) / (maximum release − spontaneous release).

Statistical analysis

Proliferation, IFN-γ secretion, and phenotype of transduced and untransduced (UT) CTLs in the absence of CN inhibitors were compared using a one-way analysis of variance (ANOVA). Proliferation and IFN-γ secretion of transduced CTLs in the presence of CN inhibitors were compared with that of UT CTLs in the absence of CN inhibitors using a one-way ANOVA with the Dunnett post hoc test. Enrichment of GFP$^+$ CTLs was assessed using the paired *t* test. Cytotoxicity was compared between transduced and UT CTLs in the presence or absence of CN inhibitors using a 2-way ANOVA. Analysis was performed using the SPSS statistical package, version 16 (SPSS).

Results

Design and generation of CN mutants

Using structural and crystallographic data from the CNa/b heterodimer in complex with FK506 or CsA, we identified critical amino acid residues involved in these interactions.[27,29,34,35] These residues were targeted in the generation of CNa or CNb mutants designed to resist binding by FK506, CsA, or both CN inhibitors. A total of 22 CNa and 32 CNb mutants were generated; the location of these mutations is illustrated in Figure 1A, and details are shown in Tables 1 and 2. A parallel approach was also investigated, whereby mutants of the FK506 carrier protein FKBP12 were generated, as shown in Figure 1B.[36] These were designed to bind FK506 but not CN, therefore sequestering the drug and preventing CN inhibition (Table 3).

CN mutants confer resistance to CN inhibitors in transient luciferase reporter assay

To assess the ability of these 54 CN mutants to confer resistance to CN inhibitors, mutants were initially assessed in a transient screening assay using Firefly and *Renilla* luciferase reporter genes. 293T cells were cotransfected with a CN mutant expression vector, an NFAT-driven Firefly luciferase (FLuc) reporter, and a constitutively expressed *Renilla* luciferase plasmid control. 293T cells were then activated with PMA

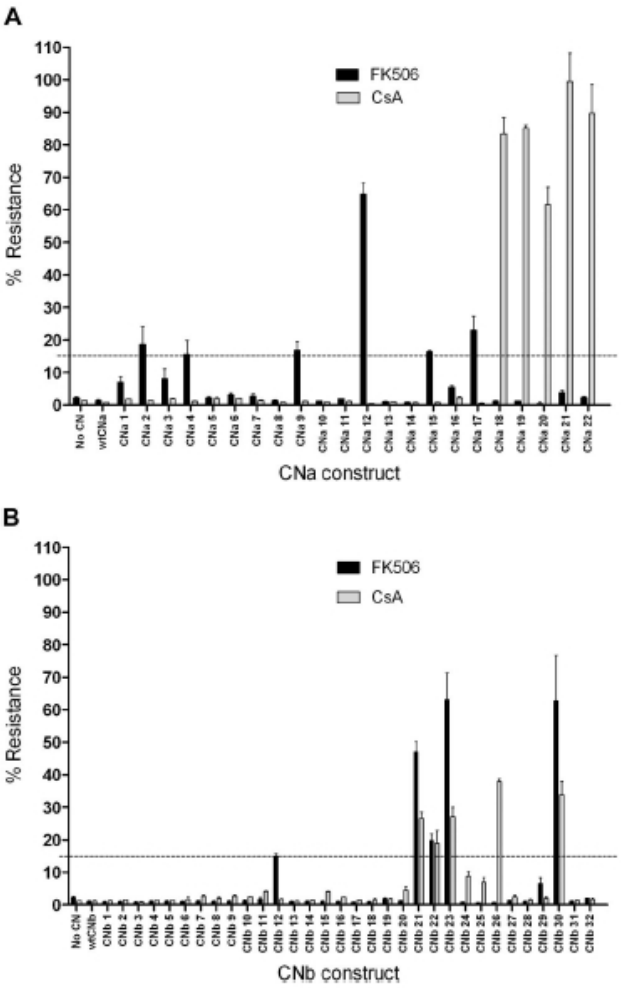

Figure 2. Transfection of 293T cells with CN mutants allows luciferase expression in the presence of CN inhibitors. 293T cells were transfected with a CN mutant, a Firefly luciferase reporter gene driven by an NFAT response element, and an internal control *Renilla* luciferase plasmid. Cells were activated with PMA and ionomycin, and the expression of luciferase was assessed. Resistance was determined by comparing luciferase expression upon stimulation in the presence of CN inhibitors to luciferase expression upon stimulation in the absence of CN inhibitors. Mean and SEM of 3 experiments shown. (A) Screening of CNa mutants identifies 6 that confer resistance to 10 ng/mL FK506 (CNa2, 4, 9, 12, 15, and 17) and 5 that confer resistance to 200 ng/mL CsA (CNa18-CNa22). (B) Screening of CNb mutants identifies 5 that confer resistance to 10 ng/mL FK506 (CNb12, 21, 22, 23, and 30) and 5 that confer resistance to 200 ng/mL CsA (CNb21, 22, 23, 26, and 30). Of the CNb mutants identified, 4 confer resistance to both FK506 and CsA (21, 22, 23, and 30).

to secrete IL-2 at 85% of this level in the presence of FK506. Similarly, transduction with CNa22 enabled IL-2 secretion in the presence of CsA (mean, 213%), and transduction with CNb30 enabled secretion of IL-2 with either FK506 (mean, 55%) or CsA (mean, 38%). The bicistronic construct of CNa12 with CNb30 allowed 101% secretion with FK506 and 79% with CsA. Based on these data, CNa12, CNa22, and CNb30 were selected for testing in EBV-CTL lines.

CN mutant transduced EBV-CTLs show a selective growth advantage in the presence of CN inhibitors

To evaluate the ability of CN mutants to confer resistance to CN inhibitors in primary cells, we tested the CN mutants identified here in EBV-CTL lines generated from 5 healthy donors. Stable retrovirus producer lines were generated to produce high-titer RD114 pseudotyped SFG retrovirus carrying the eGFP transgene alone or expressed with CNa12, CNa22, or CNb30, and supernatant from these cells was used to transduce CTLs.

Transduction efficiency was assessed before the addition of CN inhibitors and at each stimulation thereafter. Transduction efficiencies were consistently high, between 50% and 85% in all 5 donors (GFP mean, 64% [range, 54%-77%]; CNa12 mean, 64% [52%-75%]; CNa22 mean, 63% [53%-81%]; and CNb30 mean, 71% [63%-82%]). As shown in Figure 4A, EBV-CTLs transduced with GFP, CNa12, and CNb30 vectors showed stable GFP expression throughout subsequent culture when grown with no CN inhibitors, indicating that transduced CTLs do not have an intrinsic proliferative advantage over UT CTLs ($P = .239$, .24, and .309, respectively). The proportion of transduced EBV-CTLs in cultures transduced with CNa22 gradually dropped from 63% to 43% ($P = .004$), which may suggest a toxic effect.

We hypothesized that in the presence of CN inhibitors, CN mutants providing resistance should confer a selective growth advantage to transduced T cells within the CTL line, leading to an enrichment of transduced cells. This is indeed what we observed. In the presence of 10 ng/mL FK506, GFP expression remained stable in GFP and CNa22 transduced CTL lines (Figure 4B). In contrast, the proportion of GFP^+ cells rose with time in cultures transduced with CNa12 (mean, 64%-86%; $P = .002$) and CNb30 (mean, 71%-90%; $P = .01$). Similarly, as can be seen in Figure 4C in the presence of 200 ng/mL CsA, GFP expression in GFP and CNa12 transduced cultures remained stable, whereas GFP expression rose with time in

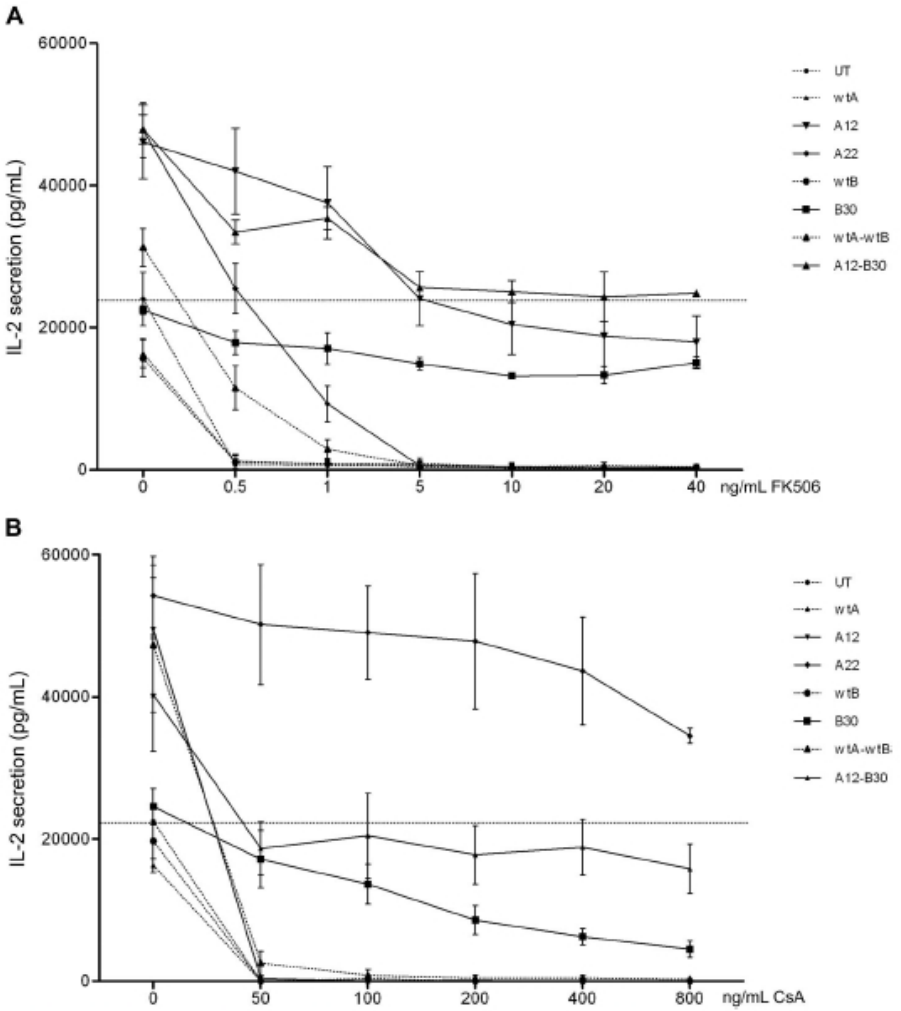

Figure 3. Jurkat cells transduced with codon-optimized CN mutants secrete IL-2 in the presence of CN inhibitors. Jurkat cells were retrovirally transduced with CN mutants and FACS sorted. Cultures were stimulated with PMA and ionomycin in the presence of increasing concentrations of FK506 (A) or CsA (B) and secretion of IL-2 assessed by ELISA. Mean and SEM of 3 experiments shown. (A) Jurkat cells transduced with CNa12, CNb30, or a bicistronic construct of these mutants were able to secrete IL-2 throughout the therapeutic range of FK506 (7-12 ng/mL). (B) Jurkat cells transduced with CNa22, CNb30, or the double construct CNa12-CNb30 were able to secrete IL-2 throughout the therapeutic range of CsA (100-250 ng/mL). Selected constructs CNa12, CNa22, CNb30, and CNa12-CNb30 are shown with solid lines; all other constructs are shown with dotted lines. The horizontal line indicates the amount of IL-2 secreted by untransduced Jurkat cells stimulated without CN inhibitors.

CNa22 transduced (mean, 63%-76%; $P = .107$) and CNb30 transduced (mean, 71%-91%; $P = .01$) CTLs. These data suggest that CTLs transduced with CN mutants have a selective advantage in the presence of the appropriate CN inhibitor, but retain similar growth kinetics as that of untransduced CTLs when grown without CN inhibitors.

Transduction of EBV-CTL lines with CN mutants allows growth in the presence of CN inhibitors

Proliferation of transduced EBV-CTL cultures was monitored for 3 weeks from addition of CN inhibitors at the fourth stimulation. In the absence of CN inhibitors, transduced CTL lines grew at a comparable rate to that of untransduced CTL lines (Figure 5A; $P = .675$). Importantly, this demonstrates that transduction with either GFP or CN mutants has no effect on the growth of EBV-CTLs.

The addition of either FK506 or CsA to EBV-CTL cultures markedly inhibited growth of both untransduced and GFP-transduced lines (Figure 5Bi and 5Bii; $P = .001$). Transduction of EBV-CTLs with CNa12 enabled growth in FK506 but not CsA (Figure 5Biii). CNa12 transduced CTLs expanded by a mean of 66-fold over 3 weeks in the absence of CN inhibitors, and 45-fold in the presence of FK506. compared with the growth of untransduced EBV-CTLs without CN inhibitors, which expanded by a mean of 85-fold, this represents 52% resistance to FK506 by CNa12 transduced EBV-CTLs ($P = .216$). CNa12 does not confer resistance to CsA ($P = .001$). As shown in Figure 5Biv, transduction with CNa22 confers resistance to CsA, allowing 90-fold expansion over 3 stimulations in the presence of CsA (106% resistance; $P > .999$) but not to FK506 ($P = .004$). Finally, transduction of EBV-CTLs with CNb30 confers resistance to both

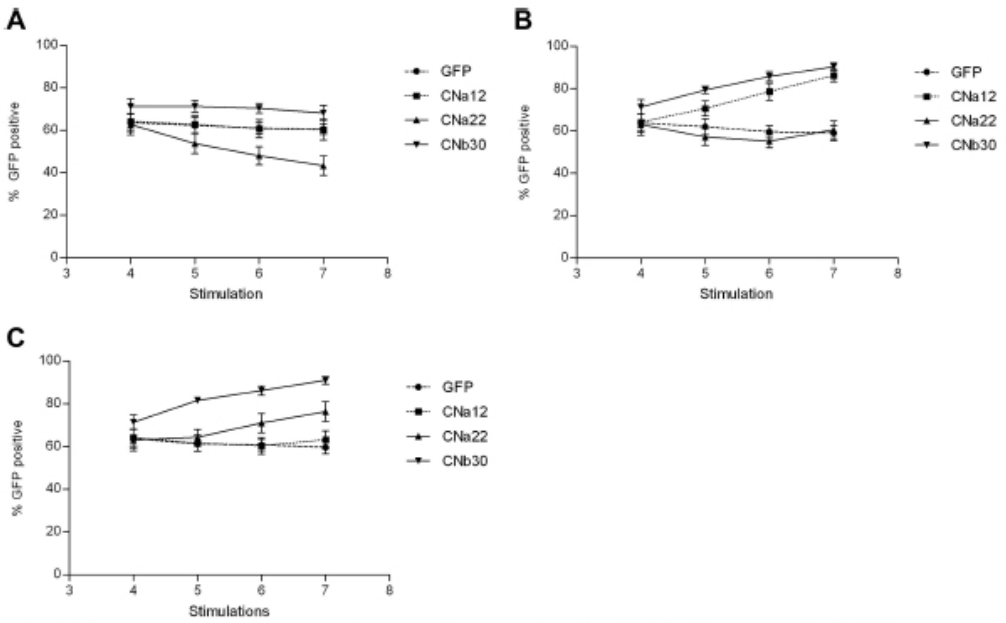

Figure 4. EBV-CTLs transduced with CN mutants show a selective advantage when cultured in FK506/CsA. Retrovirally transduced EBV-CTL lines were grown in the presence or absence of CN inhibitors for 3 weeks, and the percentage of cells expressing GFP were assessed by flow cytometry at various time points. Mean GFP expression ± SEM are shown (n = 5). (A) Expression of GFP when cultured without CN inhibitors. The proportion of transduced CTLs remains stable in all but the CNa22 culture, where the number of transduced cells drops. (B) Addition of 10 ng/mL FK506 to cultures results in an enrichment of GFP expressing CTLs in those that are resistant to FK506 (CNa12 and CNb30). (C) Addition of 200 ng/mL CsA to CTL cultures results in a rise in GFP-expressing CTLs in CsA-resistant cultures (CNa22 and CNb30).

FK506 and CsA (Figure 5Bv). In the presence of FK506, CNb30 transduced EBV-CTLs expand 57-fold (67% resistance; $P = .649$), and in the presence of CsA, 75-fold expansion is seen (88% resistance; $P > .999$).

Proliferation of transduced EBV-CTLs in the presence or absence of CN inhibitors was also assessed using ^{3}H-thymidine uptake assays after their sixth stimulation with LCLs, after 2 weeks of culture in CN inhibitors. In the absence of CN inhibitors, proliferation of all transduced CTLs was comparable with that of UT CTLs ($P = .876$). As shown in Figure 5C, the observed resistance profile in this assay was similar to that seen in Figure 5B. Untransduced or GFP transduced CTLs showed reduced proliferation in the presence of either FK506 or CsA, as previously reported.[18] In contrast, CNa12 transduced CTLs were able to proliferate in the presence of FK506 but not CsA (mean proliferation compared with untransduced CTLs in the absence of CN inhibitors was 90%/23%; $P > .999$ and $P = .02$, respectively) and CNa22 transduced CTLs showed the converse pattern (mean proliferation compared with UT controls was 26%/103%; $P = .03$/$P > .999$, respectively). Once again, transduction with CNb30 enabled CTLs to proliferate in the presence of either FK506 or CsA (mean 83%/86%; $P = .996$/$P = .999$, respectively). Furthermore, CTLs transduced with CNb30 retained the ability to proliferate in supratherapeutic levels of FK506 (40 ng/mL) and CsA (800 ng/mL; supplemental Figure 5).

Transduction with CN mutants enables cytokine secretion in the presence of CN inhibitors

To further examine the functionality of EBV-CTLs transduced with our CN mutants, we assessed the ability of such CTLs to secrete the Th1 cytokine IFN-γ in the presence of CN inhibitors. IFN-γ secretion in culture supernatants was measured 24 hours after stimulation with autologous LCL in the presence/absence of FK506/CsA. As shown in Figure 6, transduced and UT CTLs secrete similarly high levels of IFN-γ in response to stimulation with autologous LCLs ($P = .984$). In UT/GFP-transduced CTLs, this is abrogated by the addition of either 10 ng/mL FK506 or 200 ng/mL CsA ($P = .057$ and $P = .046$, respectively). In contrast, EBV-CTLs transduced with CNa12 were able to secrete IFN-γ in the presence of FK506 (mean, 66% secretion compared with untransduced CTLs in the absence of CN inhibitors [$P = .914$]), but not CsA (mean, 17% secretion; $P = .062$). Similarly, CNa22-transduced CTLs secreted IFN-γ in the presence of CsA (mean, 66%; $P = .917$), but not FK506 (mean, 16%; $P = .059$). As expected, CTLs transduced with CNb30 were able to secrete IFN-γ in the presence of either FK506 (mean, 92%; $P > .999$) or CsA (mean, 100%; $P > .999$).

Transduced EBV-CTL lines remain dependent on autologous LCL stimulation for proliferation

To ensure that CN transduced EBV-CTLs remain dependent on antigen stimulation, we examined whether transduced or UT CTLs were able to proliferate autonomously in the absence of LCL stimulation. CNb30 was chosen for these studies because it conferred resistance to both FK506 and CsA and hence is the mutant we plan to use in subsequent translational work. CTLs were cultured as normal for 4 stimulations from when LCL stimulation was withheld and CTL growth assessed. As demonstrated in Figure 7A, UT CTLs did not proliferate in the absence of stimulation with LCL. Similarly, GFP transduced and CNb30 transduced CTLs also did not grow without LCL stimulation, regardless of continued supplementation with 100 U/mL exogenous IL-2 (no LCL + IL-2, $P = .994$; no LCL/no IL-2, $P = .991$).

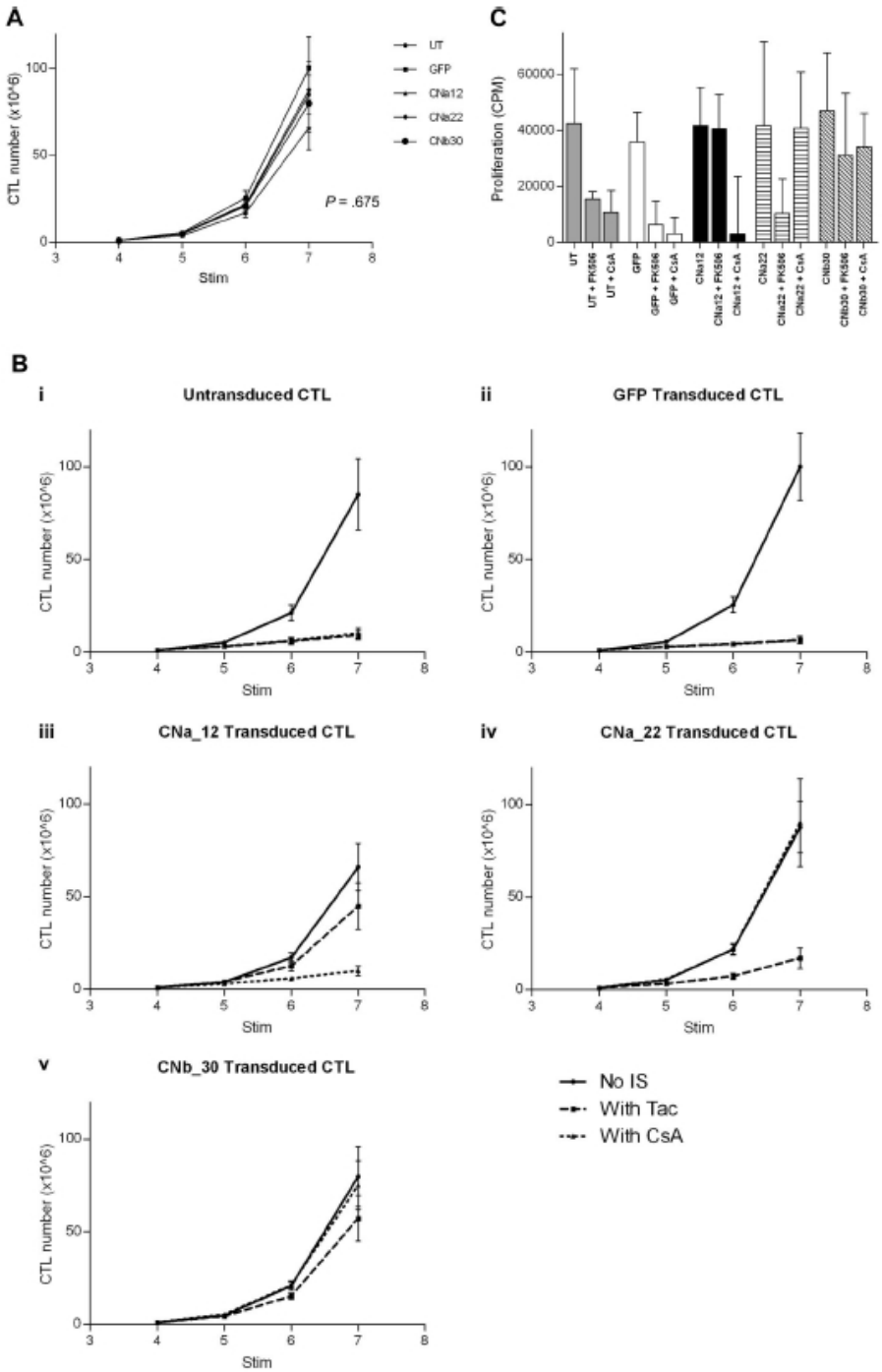

Figure 5. Transduction with CN mutants allows EBV-CTL proliferation in the presence of CN inhibitors. EBV-CTLs were transduced with either a CN mutant or with GFP control after stimulation 2. CN inhibitors were added at stimulation 4, and CTL growth was assessed for 3 weeks (mean and SEM shown, n = 5). (A) In the absence of CN inhibitors, growth of transduced CTLs and untransduced CTLs is comparable over 3 weeks ($P = .675$). (B) Expansion of EBV-CTLs over time with or without FK506/CsA. (Bi) Untransduced CTLs are suppressed by either FK506 or by CsA. (Bii) GFP-transduced CTLs are suppressed by either CN inhibitor. (Biii) CNa12-transduced CTLs proliferate in the presence of FK506 but not CsA. (Biv) CNa22-transduced CTLs proliferate in the presence of CsA but not FK506. (Bv) CNb30-transduced CTLs proliferate in the presence of either FK506 or CsA. (C) ^{3}H-thymidine uptake assay after stimulation with autologous LCLs in the presence or absence of CN inhibitors. CNa12 confers 90% resistance to FK506, CNa22 confers 103% resistance to CsA, and CNb30 confers both 83% resistance to FK506 and 86% resistance to CsA (median and interquartile range shown, n = 5).

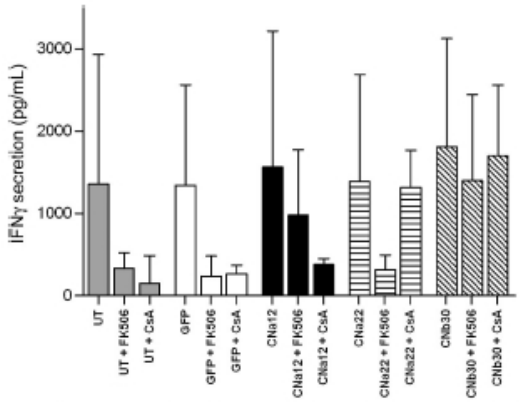

Figure 6. EBV-CTLs transduced with CN mutants secrete normal levels of IFN-γ in the presence of CN inhibitors. EBV-CTL lines cultured in the presence of CN inhibitors for 2 weeks were stimulated with autologous LCLs, and IFN-γ secretion was assessed by ELISA 24 hours after stimulation. Median and interquartile range are shown (n = 5). CTLs transduced with CNa12 were able to secrete IFN-γ in the presence of 10 ng/mL FK506 at comparable levels to that seen with untransduced CTLs in the absence of CN inhibitors (mean, 66%) and CNa22-transduced CTLs secreted IFN-γ in the presence of 200 ng/mL CsA (mean, 66%). CNb30-transduced CTLs secreted IFN-γ with either FK506 (mean, 92%) or CsA (mean, 100%).

Transduction with GFP or CNb30 does not affect the phenotype or cytotoxicity of EBV-CTL lines

To determine whether transduction with CN mutants altered the phenotype of EBV-CTL, flow cytometric analysis was performed on untransduced or CNb30 transduced CTLs 3 weeks after transduction. As shown in Figure 7B, CTLs transduced with CNb30 showed a similar phenotype to untransduced CTLs, with a predominance of $CD8^+$ $CD45RO^+$ central or effector memory T cells.

Finally, we performed cytotoxicity assays to determine the effect of retroviral transduction with CNb30 on the cytotoxic activity of EBV-CTL lines. To assess this, ^{51}Cr release cytotoxicity assays were performed after 2 to 3 weeks of culture in 10 ng/mL FK506 or 200 ng/mL CsA (6 days after stimulation 5 or 6). As illustrated in Figure 7Ci, in the absence of immunosuppression, cytotoxicity against autologous LCL targets was similar in untransduced, GFP transduced, and CNb30 transduced CTLs (mean, 24.6%, 24.7%, and 26.2%, respectively, at an effector-target ratio of 30:1; $P = .811$). Prior culture of CTLs in either FK506 (Fig 7Cii) or CsA (Fig 7Ciii), and inclusion of these in the cytotoxicity assay, did not significantly affect cytotoxic activity against autologous LCLs. Neither untransduced or transduced CTLs showed significant cytotoxicity against allogeneic LCLs, confirming that CTL lines are EBV specific and MHC restricted, and that transduction with our CN mutants does not increase alloreactivity (UT mean, 6.5%; GFP, 5%; CNb30, 6.9% at effector-target ratio of 30:1; $P = .811$). These data demonstrate that the cytotoxicity of EBV-CTL lines is not affected by either retroviral transduction with CNb30, or by the presence of therapeutic doses of CN inhibitors.

Discussion

Adoptive immunotherapy with EBV-specific CTLs has been given to more than 200 patients and is a highly effective strategy for both treatment and prophylaxis for PTLD after SCT.[37] However, clinical studies using EBV-CTLs after SOT have been disappointing, most likely due to the inhibitory effect of ongoing pharmacologic immunosuppression on infused CTLs.[19] To address this problem, we engineered EBV-CTLs to be resistant to suppression with CN inhibitors and have identified 3 CN mutants which enable EBV-CTLs to function in the presence of these drugs: CNa12 renders EBV-CTLs resistant to FK506 and CNa22 renders EBV-CTLs resistant to CsA, whereas CNb30 confers resistance to both.

Binding of FK506 and CsA to their chaperone proteins FKBP12 and CyPA, respectively, leads to docking of these small-molecule/protein complexes with the CN heterodimer, sterically blocking entry and subsequent activation of NFAT. The interactions between these complexes and CN occur at a common site at the CNa/CNb interface. This site is distinct from the CN active site, allowing design of mutations which inhibit docking of either or both FK506/FKBP12 and CsA/CyPA complexes, but which do not affect NFAT dephosphorylation. CNa12 was based on a novel combination of mutations first identified by Kawamura;[28] we altered CNa by introducing substitutions T351E and L354A. These mutations should disrupt binding between CNa and the charged surface residues H87-P88 of FKBP12,[38] but should not affect CsA/CyPA binding. CNa22 contains the mutations V314R, which directly disrupts CsA binding and Y341F,[35] which could prevent FKBP12/CyPA binding by steric inhibition of close contact with the body of CNa. CNb30 has both the point mutation L124T[39] and the insertion K125-LA in the latch region (based on the naturally occurring K125-VQ[40]) disrupting the folding structure, which is involved in binding of CNb to CNa but also in binding of FKBP12/CyPA to the CN heterodimer.[39]

Growth of transduced and untransduced CTL lines was assessed in the presence of therapeutic doses of either FK506 or CsA. As expected, addition of either to untransduced or control GFP transduced EBV-CTLs inhibited proliferation. We compared the growth of EBV-CTLs without CN inhibitors over a 3-week culture period with that of EBV-CTLs transduced with CN mutants in the face of therapeutic doses of either FK506 or CsA. CNa12 transduced CTLs proliferated at rates comparable with that of control CTLs in the presence of 10 ng/mL FK506, CNa22 transduced CTLs proliferated well in the presence of 200 ng/mL CsA, and EBV-CTLs transduced with CNb30 were resistant to suppression by either FK506 or CsA. Further, we have also demonstrated that these transduced EBV-CTLs retain functionality against EBV-infected target cells in the presence of CN inhibitors: whereas secretion of IFN-γ in response to LCL stimulation by untransduced EBV-CTLs was abrogated by either CsA or FK506, transduced CTLs showed equivalent secretion of IFN-γ in the presence or absence of CN inhibitors in the same pattern as seen with our proliferation data. Finally, we have shown that transduction with CNb30 (our favored construct) has no effect on either the phenotype or cytotoxic activity of EBV-CTLs. In particular, CNb30 transduced CTLs showed no increase in alloreactivity in cytotoxicity assays.

The CN heterodimer is a pivotal signal transduction molecule in mediating transcriptional activation of T cells after calcium flux from T-cell receptor (TCR) engagement. One potential unwanted consequence of our engineering is autonomous or aberrant activity of a mutant CN. Alternatively, simple overexpression of CN might lead to hyperactivation of transduced cells after stimulation. In transduced Jurkat cells, none of the 54 mutant CNs allowed autonomous IL-2 secretion; however some increased production of IL-2 from CN-transduced cultures was observed upon stimulation with PMA and ionomycin compared with UT control. This effect

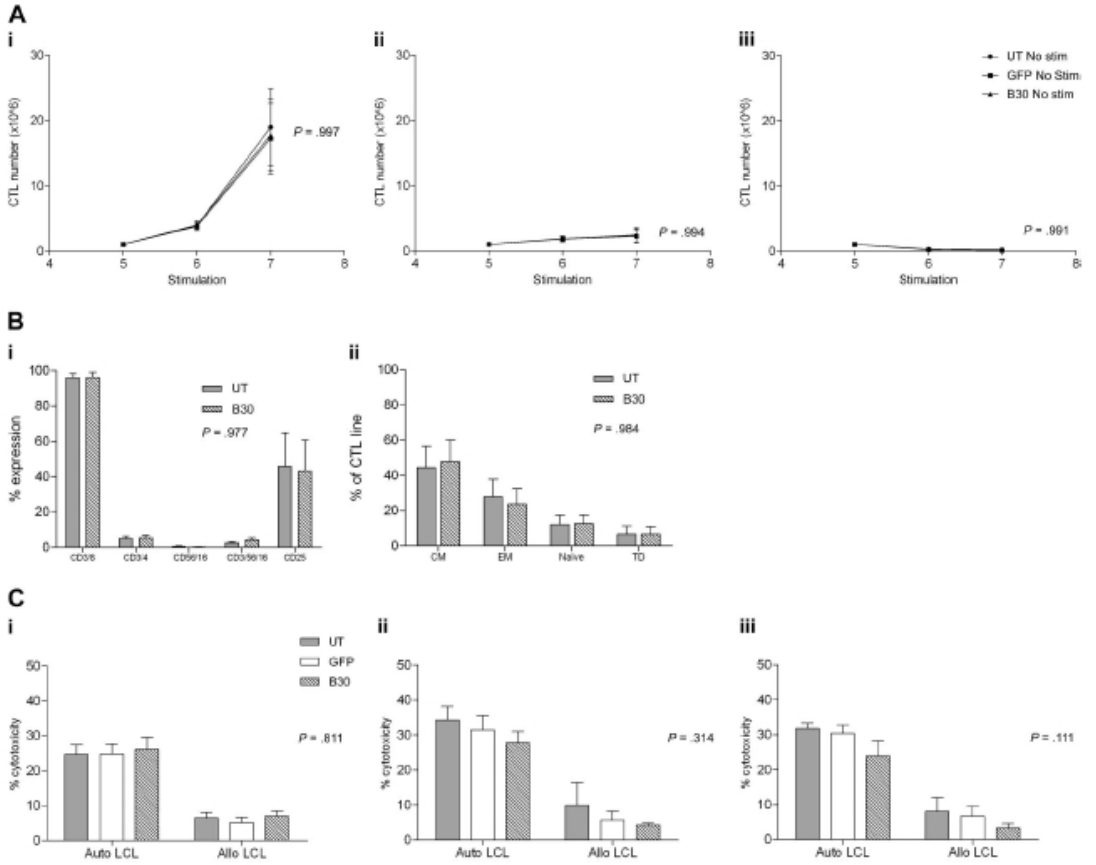

Figure 7. Antigen dependence, phenotype, and cytotoxicity of EBV-CTLs are unaffected by transduction with CNb30. (A) Transduced and untransduced EBV-CTLs were grown as normal for 4 stimulations. Proliferation was monitored from the day of stimulation 5. Mean and SEM shown (n = 5). (Ai) Growth of CTLs continuing to receive weekly stimulation with autologous LCLs and exogenous IL-2. (Aii) CTLs that receive no LCL stimulation do not proliferate, even with continuing supplement of 100 U/mL IL-2. (Aiii) CTLs that do not receive LCL stimulation or IL-2 do not proliferate. There is no difference in proliferation between transduced and untransduced CTL lines under any of these conditions. (B) EBV-CTLs were assessed for expression of surface markers 4 days after their fifth stimulation with autologous LCLs. Mean and SEM shown (n = 5). (Bi) Percentage of expression of CD3, CD4, CD8, CD16/56, and CD25 in untransduced and CNb30-transduced lines. (Bii) Distribution of memory subsets in untransduced and CNb30-transduced lines. CM indicates central memory ($CD45RO^+$, $CD62L^+$); EM, effector memory ($CD45RO^+$, $CD62L^-$); naive ($CD45RO^-$, $CD62L^+$); and TD, terminally differentiated ($CD45RO^-$, $CD62L^-$). (C) A ^{51}Cr release cytotoxicity assay was performed to assess cytotoxicity against autologous or allogeneic LCL targets of EBV-CTL lines cultured for 2 to 3 weeks in the presence or absence of CN inhibitors (n = 5). Mean and SEM cytotoxicity at an effector-target ratio of 30:1 are shown. (Ci) After culture in the absence of CN inhibitors. (Cii) After 2 to 3 weeks of culture in 10 ng/mL FK506. (Ciii) After 2 to 3 weeks of culture in 200 ng/mL CsA. No effect of CN inhibitors was detected on cytotoxicity of transduced or untransduced EBV-CTLs. CN inhibitors were present during the cytotoxicity assay in panels Cii and Ciii.

was more pronounced in cells transduced with CNa than CNb, which is likely to reflect CNa as the limiting component of the CNa/CNb heterodimer[39] (supplemental figure 2). However, in primary EBV-CTLs, expression of CN mutants had no effect on cell proliferation in the absence of CN inhibitors (Figure 5A) with or without stimulation. Importantly, no EBV-CTL expansion was observed upon removal of LCL stimulation in transduced lines even when supplemented with exogenous IL-2, confirming that proliferation remains entirely antigen-dependent.

Generation of CTLs that can function in the presence of immunosuppression represents a considerable advance for the immunotherapy of PTLD and may enable extension of the benefits of this approach to the SOT setting. Potentially, adoptive transfer of autologous CN inhibitor–resistant EBV-specific CTLs could be used as prophylaxis for PTLD in high-risk groups, such as in patients undergoing pediatric small-bowel transplantation, where the risk of PTLD may be as high as 30%.[41] In cohorts at lower risk of PTLD, resistant EBV-CTLs could be used as adjunctive therapy for established PTLD with rituximab. In this situation, first-line therapy with rituximab could be used to establish disease control during the time required for generation of the EBV-CTLs, with subsequent transfer of resistant CTLs to maintain remission and overcome the significant rates of partial response and relapse associated with rituximab monotherapy.[42-44] Critically, such a strategy would obviate the need for reduction in immunosuppression with calcineurin inhibitors, which, as noted, is a frequent cause of rejection and treatment failure.[4] Importantly, the methodology we used has previously been used to generate therapeutic cell products for more than 100 patients; therefore, we are confident these EBV-CTLs will have clinical efficacy. As a prelude to such clinical studies, we are currently testing the efficacy of EBV-CTLs transduced with CNb30 in preventing or treating EBV–lymphoproliferative disease (LPD) in the presence of CN inhibitors in a xenogeneic γ-chain$^-$/RAG2$^-$/C5$^-$ mouse model.[45,46]

Other factors may limit the effectiveness of resistant EBV-CTLs to treat PTLD in the SOT setting. Firstly, the efficacy of infused EBV-CTLs after SCT may have been enhanced by the availability of an immunologic niche due to the lymphodepletion caused by conditioning, a situation clearly different from SOT. However, EBV-CTLs have proven functional without prior lymphodepletion.[47] In addition, the selective advantage-resistant EBV-CTLs have in an immunosuppressed host may result in effects equivalent to that of pharmacologic conditioning. Second, the requirement for continued antigen stimulation to promote survival of some types of adoptively transferred T cells may limit their use as long-term prophylaxis or treatment. In fact, EBV-CTLs generated as we have have shown remarkable long-term survival in vivo: gene-marked EBV-CTLs have been detected in peripheral blood of patients many years after infusion, often increasing in response to rise in EBV viral load.[48] Finally, although CN inhibitors form the cornerstone of immunosuppressive regimens in SOT, these agents are often combined with others, particularly mycophenolate (MMF). This agent is likely dispensable in the face of PTLD. If resistant EBV-CTLs are to be given as prophylaxis in a clinical setting where MMF is indispensible, it should be possible to coexpress a previously described mutant IMPDH2 that will render T cells resistant to MMF[49] in addition to FK506/CyA.

Our approach represents a generic means of enabling adoptively transferred T cells to function in the face of ongoing immunosuppression and has important broader applicability. Potentially, this could be used to enable adoptively transferred T cells with specificity for other viruses to function despite ongoing immunosuppression in either the SCT or SOT settings. Similarly, our approach may enable T cells redirected to recognize leukemia-associated antigens or cell-surface molecules by TCR[50] or chimeric antigen receptor[51] (CAR) transfer to be administered early after SCT, without the need for withdrawal of immunosuppression and the attendant risk of graft-versus-host disease (GVHD). In this regard, the small size of our preferred mutant CNb30 (approximately 500 bp) will allow coexpression of other useful transgenes, including TCRs, CARs, other resistance genes (eg, IMPDH2),[49] and suicide genes.[52] Potentially, administration of calcineurin inhibitors after transfer of T cells transduced with our mutants may enable in vivo selection for transduced cells expressing these other transgenes. Further, there may be clinical utility to using a CN mutant which renders EBV-CTLs resistant to only one CN inhibitor, because these CTLs could be suppressed using the alternative CN inhibitor if adverse effects were observed.

In summary, we report the generation of CN mutants that confer resistance to either or both FK506 and CsA. EBV-CTLs transduced with these mutants retain the capacity to proliferate, secrete cytokines, and kill target cells in the presence of therapeutic levels of CN inhibitors. These resistant T cells could increase the efficacy of immunotherapy for PTLD after SOT, and may have wider applications for immunotherapy in other settings.

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Authorship

Contribution: J.B., M.P. and P.J.A. designed the work; J.B. and M.P. designed and generated the CN mutants; M.P. codon-optimized CNa and CNb; J.B. performed experiments and analyzed data; C.M., K.S., H.K., and S.S. gave assistance in the laboratory; J.B., M.P., and P.J.A. wrote the manuscript; and all authors read and commented on the paper.

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References

1. Lim WH, Russ GR, Coates PT. Review of Epstein-Barr virus and post-transplant lymphoproliferative disorder post-solid organ transplantation [review]. *Nephrology*. 2006;11(4):355-366.
2. Opelz G, Dohler B. Lymphomas after solid organ transplantation: a collaborative transplant study report. *Am J Transplant*. 2004;4(2):222-230.
3. Pinkerton CR, Hann I, Weston CL, et al. Immunodeficiency-related lymphoproliferative disorders: prospective data from the United Kingdom Children's Cancer Study Group Registry. *Br J Haematol*. 2002;118(2):456-461.
4. Webber SA, Naftel DC, Fricker FJ, et al. Lymphoproliferative disorders after paediatric heart transplantation: a multi-institutional study. *Lancet*. 2006;367(9506):233-239.
5. Gross TG, Bucuvalas JC, Park JR, et al. Low-dose chemotherapy for Epstein-Barr virus-positive post-transplantation lymphoproliferative disease in children after solid organ transplantation. *J Clin Oncol*. 2005;23(27):6481-6488.
6. Hanto DW, Frizzera G, Gajl-Peczalska KJ, Simmons RL. Epstein-Barr virus, immunodeficiency, and B cell lymphoproliferation. *Transplantation*. 1985;39(5):461-472.
7. Hsieh WS, Lemas MV, Ambinder RF. The biology of Epstein-Barr virus in post-transplant lymphoproliferative disease. *Transpl Infect Dis*. 1999;1(3):204-212.
8. Rickinson AB, Lee SP, Steven NM. Cytotoxic T lymphocyte responses to Epstein-Barr virus. *Curr Opin Immunol*. 1996;8(4):492-497.
9. Hislop AD, Taylor GS, Sauce D, Rickinson AB. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol*. 2007;25:587-617.
10. Rooney CM, Smith CA, Ng CY, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood*. 1998;92(5):1549-1555.
11. Rooney CM, Smith CA, Ng CY, et al. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet*. 1995;345(8941):9-13.
12. Gustafsson A, Levitsky V, Zou JZ, et al. Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. *Blood*. 2000;95(3):807-814.
13. Hale GA, Pule M, Amrolia P, et al. Long-term follow-up of administration of donor-derived EBV-specific CTLs to prevent and treat EBV lymphoma after hemopoietic stem cell transplant. *Biol Blood Marrow Transplant*. 2006;14(2):3.
14. Savoldo B, Goss JA, Hammer MM, et al. Treatment of solid organ transplant recipients with autologous Epstein Barr virus-specific cytotoxic T lymphocytes (CTLs). *Blood*. 2006;108(9):2942-2949.
15. Comoli P, Labirio M, Basso S, et al. Infusion of autologous Epstein-Barr virus (EBV)–specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication. *Blood*. 2002;99(7):2592-2598.
16. Khanna R, Bell S, Sherritt M, et al. Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. *Proc Natl Acad Sci U S A*. 1999;96(18):10391-10396.
17. Haque T, Wilkie GM, Jones MM, et al. Allogeneic cytotoxic T-cell therapy for EBV-positive post-transplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood*. 2007;110(4):1123-1131.
18. Savoldo B, Goss J, Liu Z, et al. Generation of autologous Epstein-Barr virus-specific cytotoxic T cells for adoptive immunotherapy in solid organ transplant recipients. *Transplantation*. 2001;72(6):1078-1086.
19. Zhan X, Brown B, Slobod KS, Hurwitz JL. Inhibition of ex vivo-expanded cytotoxic T-lymphocyte function by high-dose cyclosporine. *Transplantation*. 2003;76(4):739-740.
20. Engstrand M, Lidehall AK, Totterman TH, Herrman B, Eriksson BM, Korsgren O. Cellular

responses to cytomegalovirus in immunosuppressed patients: circulating CD8+ T cells recognizing CMVpp65 are present but display functional impairment. *Clin Exp Immunol.* 2003; 132(1):96-104.

21. Tsai DE, Hardy CL, Tomaszewski JE, et al. Reduction in immunosuppression as initial therapy for posttransplant lymphoproliferative disorder: analysis of prognostic variables and long-term follow-up of 42 adult patients. *Transplantation.* 2001;71(8):1076-1088.
22. Kirk ADMRB, Swanson SJ, Hale DA. Strategies for minimizing immunosuppression in kidney transplantation. *Transpl Intl.* 2005;18(1):2-14.
23. Im SH, Rao A. Activation and deactivation of gene expression by Ca2+/calcineurin-NFAT-mediated signaling. *Mol Cells.* 2004;18(1):1-9.
24. Ho S, Clipstone N, Timmermann L, et al. The mechanism of action of cyclosporin A and FK506. *Clin Immunol Immunopathol.* 1996;80(3):S40-S45.
25. Griffith JP, Kim JL, Kim EE, et al. X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell.* 1995;82(3):507-522.
26. Zhu DH, Cardenas ME, Heitman J. Calcineurin mutants render T lymphocytes resistant to cyclosporin A. *Mol Pharmacol.* 1996;50(3):506-511.
27. Ke H, Huai Q. Structures of calcineurin and its complexes with immunophilins-immunosuppressants. *Biochem Biophys Res Commun.* 2003;311(4): 1095-1102.
28. Kawamura A, Su MS. Interaction of FKBP12-FK506 with calcineurin A at the B subunit-binding domain. *J Biol Chem.* 1995;270(26):15463-15466.
29. Clipstone NA, Fiorentino DF, Crabtree GR. Molecular analysis of the interaction of calcineurin with drug-immunophilin complexes. *J Biol Chem.* 1994;269(42):26431-26437.
30. Heckman KL, Pease LR. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc.* 2007;2(4):924-932.
31. Riviere I, Brose K, Mulligan RC. Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. *Proc Natl Acad Sci U S A.* 1995; 92(15):6733-6737.
32. Kelly PF, Carrington J, Nathwani A, Vanin EF. RD114-pseudotyped oncoretroviral vectors: biological and physical properties. *Ann N Y Acad Sci.* 2001;938:262-276.
33. Cosset FL, Takeuchi Y, Battini JL, Weiss RA, Collins MK. High-titer packaging cells producing recombinant retroviruses resistant to human serum. *J Virol.* 1995;69(12):7430-7436.
34. Jin L, Harrison SC. Crystal structure of human calcineurin complexed with cyclosporin A and human cyclophilin. *Proc Natl Acad Sci U S A.* 2002; 99(21):13522-13526.
35. Cardenas ME, Muir RS, Breuder T, Heitman J. Targets of immunophilin-immunosuppressant complexes are distinct highly conserved regions of calcineurin-A. *EMBO J.* 1995;14(12):2772-2783.
36. Barik S. Immunophilins: for the love of proteins. *Cell Mol Life Sci.* 2006;63(24):2889-2900.
37. Merlo A, Turrini R, Dolcetti R, Zanovello P, Amadori A, Rosato A. Adoptive cell therapy against EBV-related malignancies: a survey of clinical results. *Expert Opin Biol Ther.* 2008;8(9): 1265-1294.
38. Aldape RA, Futer O, DeCenzo MT, Jarrett BP, Murcko MA, Livingston DJ. Charged surface residues of FKBP12 participate in formation of the FKBP12-FK506-calcineurin complex. *J Biol Chem.* 1992;267(23):16029-16032.
39. Milan D, Griffith J, Su M, Price ER, McKeon F. The latch region of calcineurin B is involved in both immunosuppressant-immunophilin complex docking and phosphatase activation. *Cell.* 1994; 79(3):437-447.
40. Fox DS, Cruz MC, Sia RA, et al. Calcineurin regulatory subunit is essential for virulence and mediates interactions with FKBP12-FK506 in *Cryptococcus neoformans*. *Mol Microbiol.* 2001;39(4): 835-849.
41. Finn L, Reyes J, Bueno J, Yunis E. Epstein-Barr virus infections in children after transplantation of the small intestine. *Am J Surg Pathol.* 1998;22(3): 299-309.
42. Choquet S, Leblond V, Herbrecht R, et al. Efficacy and safety of rituximab in B-cell post-transplantation lymphoproliferative disorders: results of a prospective multicenter phase 2 study. *Blood.* 2006;107(8):3053-3057.
43. Choquet S, Oertel S, LeBlond V, et al. Rituximab in the management of post-transplantation lymphoproliferative disorder after solid organ transplantation: proceed with caution. *Ann Hematol.* 2007;86(8):599-607.
44. Messahel B, Taj MM, Hobson R, et al. Single agent efficacy of rituximab in childhood immunosuppression related lymphoproliferative disease: a United Kingdom Children's Cancer Study Group (UKCCSG) retrospective review. *Leuk Lymphoma.* 2006;47(12):2584-2589.
45. Nabarro S, Himoudi N, Papanastasiou A, et al. Coordinated oncogenic transformation and inhibition of host immune responses by the PAX3-FKHR fusion oncoprotein. *J Exp Med.* 2005; 202(10):1399-1410.
46. Lacerda JF, Ladanyi M, Louie DC, Fernandez JM, Papadopoulos EB, O'Reilly RJ. Human Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes home preferentially to and induce selective regressions of autologous EBV-induced B cell lymphoproliferations in xenografted C.B-17 scid/scid mice. *J Exp Med.* 1996;183(3):1215-1228.
47. Pule MA, Savoldo B, Myers GD, et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med.* 2008;14(11):1264-1270.
48. Heslop HE, Slobod KS, Pule MA, et al. Long-term outcome of Epstein Barr virus–specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood.* Prepublished October 2009 as DOI:10.1182/blood-2009-08-239186.
49. Yam P, Jensen M, Akkina R, et al. Ex vivo selection and expansion of cells based on expression of a mutated inosine monophosphate dehydrogenase 2 after HIV vector transduction: effects on lymphocytes, monocytes, and CD34+ stem cells. *Mol Ther.* 2006;14(2):236-244.
50. Xue SA, Gao L, Hart D, et al. Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. *Blood.* 2005;106(9):3062-3067.
51. Pule M, Finney H, Lawson A. Artificial T-cell receptors. *Cytotherapy.* 2003;5(3):211-226.
52. Straathof KC, Pule MA, Yotnda P, et al. An inducible caspase 9 safety switch for T-cell therapy. *Blood.* 2005;105(11):4247-4254.